Studies of cytoadherence and *var* gene transcription in *Plasmodium falciparum*

George Thomas Clottey

A thesis submitted to the University of Edinburgh in part fulfilment of the requirement for the degree of

Doctor of Philosophy

Institue of Cell, Animal and Population Biology

January 2001

Abstract

Studies of cytoadherence and *var* gene transcription in *Plasmodium falciparum*

George Thomas Clottey

Submitted towards the degree of Doctor of Philosophy, University of Edinburgh, January 2001

Plasmodium falciparum infection can be distinguished from that of other plasmodia which infect humans by one specific facet of it's biology, that of sequestration of mature developmental forms in capillary beds. A variant antigen, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) is involved in this process and members of this trypsin soluble 210-230 kDa protein family are responsible for cytoadherence of mature infected erythrocytes. Their sequestration in vital organs including the brain, lungs, kidney and placenta may result in local pathology and in severe malaria syndromes such as cerebral malaria. PfEMP-1 genes are also subject to clonal antigenic variation and the development of anti-PfEMP-1 antibody may be a highly significant component of immunity against both mild and severe malaria.

A model system has been developed to investigate the molecular basis of infected erythrocyte adhesion to host endothelial receptors. *P. falciparum* clone 3D7A was selected for adhesion to Chinese Hamster Ovary (CHO) cells resulting in an adhesive phenotype, which is sensitive to trypsin treatment of infected erythrocytes. The nature of the cytoadesion receptor on the CHO cell surface has been analysed and does not appear to correspond to any of the protein or glycoprotein cytoadherence receptors so far described. It has been demonstrated that unselected 3D7A transcribes at least 27 different *var* genes. On selection for adhesion to CHO cells this transcription is narrowed down to the transcription of a single gene named *var*-CHO. This gene has been cloned, sequenced and annotated. *Var*-CHO consists of 3 DBL domains, DBL-α, DBL-β and DBL-δ, two CIDR domains, CIDR-α and CIDR-β, and a C2 domain. A provisional genomic map position of this member of the PfEMP-1 has been identified as chromosome 13 by a combination of pulse field gel and *P. falciparum* genome project data.

"Success should be judged not on one's achievements, but on what one has overcome in order to achieve them"

Anonymous

Declaration

I declare that the work presented in this thesis is work of my own composition and that it has not been submitted to any other university or for any other degree.

Dedication

I dedicate this thesis to my parents, Fred and Mercy Clottey, whom have made immeasurable sacrifice in order for me to achieve my goals.

Acknowledgements

Where do I begin? I would like to thank my primary school teacher Miss Bloom for.....only joking!!!. I would like to thank my supervisors Dr David Arnot and Prof. David Walliker. Dr Arnot for introducing me to molecular biology, I appreciate your "hands off" approach to supervision, permitting the development of my thinking, whilst providing support from afar. Prof. David Walliker for welcoming me into the Edinburgh malaria group, I always felt that your door was open to me, and greatly value our discussions.

I would also like to thank my colleagues in the Arnot lab, My "big sis" Mercy Sowa, for technical advice, hours of counselling, all round support and great cooking, Alison Creasey, for allowing me tap Into your wealth of knowledge in malaria culture, and for patience in reading my thesis, Amel Hamed, what can I say, my view of Sudanese music will never be the same again and Chris Ward, thanks for the diploma in "Glaswegian English", your Glasgow humour will not be forgotten, 24 Carat Glaswegian Gold.

Others in the department I must thank include the Blaxter lab, for tolerating my excessive compulsive dongle theft. David Guliano for genomics advice, Aziz Aboobaker, for being a friend and comrade, you made Edinburgh a fun place to be. Alex Rowe, our resident *var* gene guru, thanks for the discussions and scientific input, I almost felt like a real scientist for a while. Special thanks to Prof. Kevin Marsh, for introducing me to malaria, and giving me confidence in my ability, and my fellow South Londoner, Dr Robert Snow, for being an inspiration. Self confidence, ability, and hard work indeed translate into success, irrespective of one's background.

Thanks to my parents for all their love support, I hope I've done you proud. My brothers Sonny and Russell, I know that you have never really appreciated why I've spent all these years in academia, but I know you're happy for me to have achieved my goals. My little sister Diana, be inspired. The Dan-Nartey family, Mrs Dan-Nartey for good fried chicken and Mr Dan-Nartey for many words of wisdom.

Tony Graham, thanks for "having my back" over the last 20+ years, and for "keeping it real". More could be said, but there just isn't the space.

Last but not least, Lisa Dan-Nartey, this would not have been possible with out your support, I love you.

Introduction

Chapter 2

Materials and Methods

Chapter 3

Development of a model system for studying cytoadhesion of *P.falciparum* infected red blood cells: selecting 3D7A lines which bind Chinese Hamster Ovary Cells

Chapter 4

Characterisation of PfEMP-1/*var* gene transcription in unselected 3D7A cultures

Chapter 7

Tables

List of Abbreviations:

amp.	ampicillin
bp	base pairs
°C	degree centigrade
CIDR	cysteine-rich interdomain region
CSA	chondroitin sulphate A
CSC	chondroitin sulphate C
CSP	circumsporozoite protein
DBL	Duffy-binding like domain
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide diphosphates
EBA175	erythrocyte-binding antigen 175
EDTA	ethylenediamine N,N,N',N',tetra-acetic acid
ECAM-1	endothelial cell adhesion molecule
g	gram
GPI	glycophosphatidylinositol
GST	glutathione-S-transferase
h	hour
HA	Hyaluronic acid
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphoic acid)
HS	Heparan sulphate
IFN_	interferon alpha
IL2	interleukin 2
kb	kilobase
kDa	kilodalton
IPTG	isopropyl-β-D-thiogalactoside
L	litre
LB	Luria-Bertani medium
M	molar
Mb	megabase
mg	milligram
ml	millilitre
mM	millimolar
mg	microgram
ml	microlitre
Mm	micromolar
min	minute
MSP1	merozoite surface protein 1
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PECAM	platelet/endothelial cell adhesion molecule
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
rpm	revolutions per minute

RPMI	Rosewell Memorial Park Institute
RNA	ribonucleic acid
SBEC	saimiri brain endothelial cells
SDS	sodium dodecyl sulphate
TAE	tris-acetate-EDTA
TNF-α	tumour necrosis factor alpha
Tris	Tris(hydroxymethyl) aminoethane
VCAM-1	vascular cell adhesion molecule type 1
WHO	World Health Organisation
w/v	weight per volume
xGal	5-bromo-4-chloro-3-indolyl-4-D-galactopyranoside

Introduction

1.1 The biology of malaria: the parasite life cycle

The elucidation of the life cycle of the malaria parasite ranks as one of the greatest advancements of medical science. Starting with Laveran's description of the exflagellation of microgametes in a blood sample taken from a French soldier serving in Algeria (Laveran 1880), and climaxing with the work of Grassi and Ross and the definitive description of the *Anopheles* mosquito as the vector infecting humans in 1898-99. The identification of liver forms as the intermediate stage between sporozoite infection and the erythrocytic invasion cycle by Shortt & Garnham (1948) ended an era of biological research which defined key features of the parasite and which subsequently had major influences on the epidemiology of the disease. Our current understanding of the life cycle is as follows.

Human infection by *Plasmodium falciparum* is initiated by the inoculation of sporozoites from the salivary glands of a female *Anopheles* mosquito into subcutaneous capillaries. After invading the parenchymal cells of the liver each sporozoite starts to replicate via a process known as hepatic schizogony (Figure 1.1, Phase 3). The nucleus divides repeatedly generating up to 30,000 merozoites in each hepatic schizont. Hepatic schizogony terminates as exoerythrocytic merozoites are released by schizont rupture. This is followed by merozoite invasion of erythrocytes in the blood stream. Invasion is achieved by the attachment of the merozoite apical complex to the erythrocyte

membrane, formation of a vacuole and engulfment of the merozoite by the erythrocyte. Inside the erythrocyte the merozoite becomes vacuolated and ring shaped, becoming a so-called ring form. Growth of the ring form results in the parasite occupying a large portion of the cell and this growing stage is thus called a trophozoite. This stage undergoes nuclear division, which continues until the parasite reaches maturity with the formation of 8-16 erythrocytic merozoites by a process known as erythrocytic schizogony (Figure 1.1, Phase 4,). The erythrocyte ruptures and the merozoites are released into circulation. Many are destroyed by the host immune system, but others survive and re-invade other erythrocytes, perpetuating another cycle of erythrocytic schizogony. In each cycle a small proportion of the intracellular merozoites do not become schizonts but develop into gametocytes which are taken up into the mosquito during a blood meal, where zygote formation takes place. The zygote becomes an ookinete which invades the gut wall to form oocysts (Figure 1.1, Phase 1). The oocysts eventually burst to release sporozoites, some of which re-invade the salivary glands (Figure 1.1, Phase 2). The mosquito injects saliva, containing sporozoites, while obtaining a blood meal from a new host, thus perpetuating further cycles of parasite reproduction.

.

Figure 1.1 Parasite Life Cycle

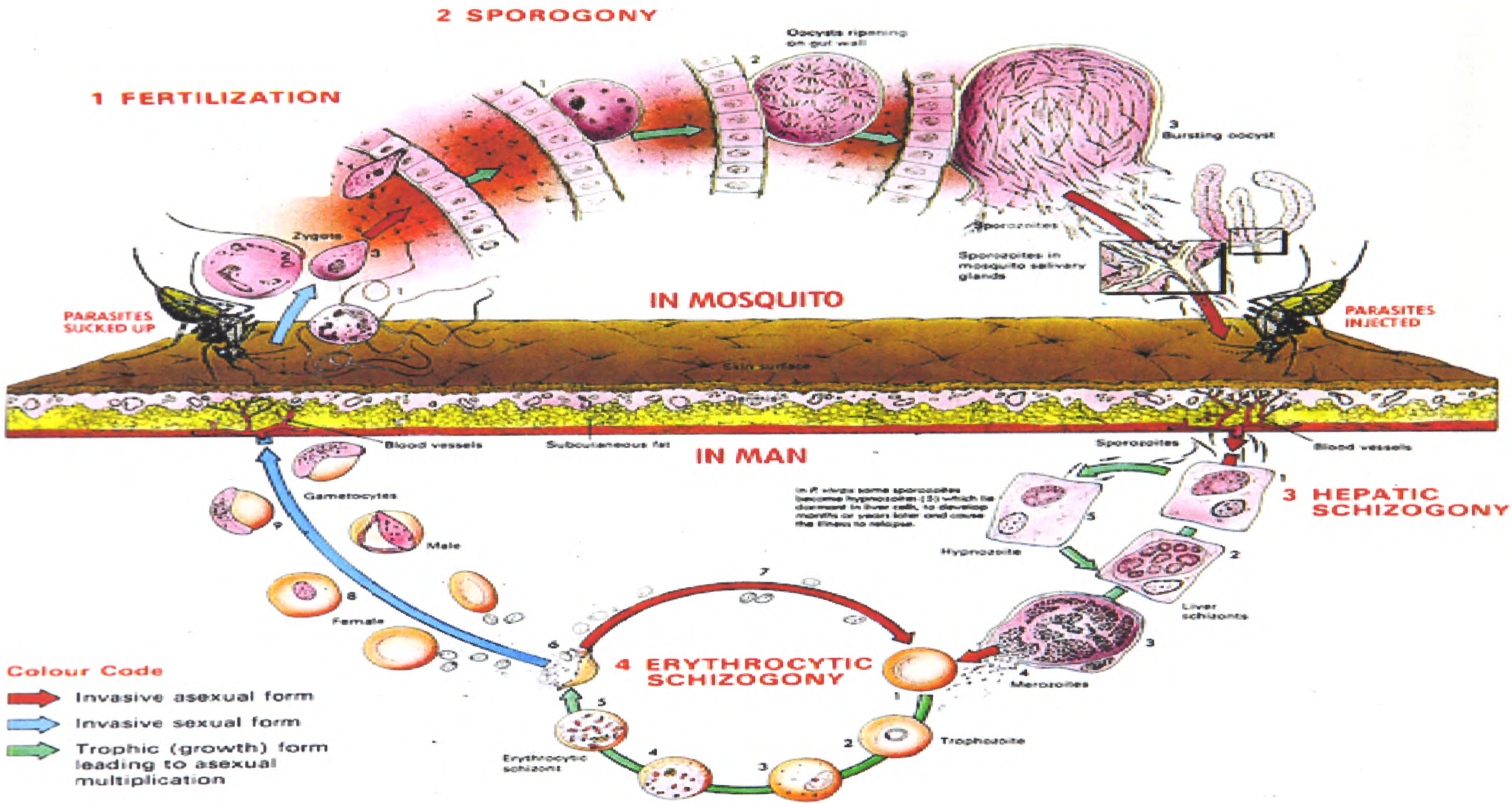

Figure 1.1 shows the Life cycle of Plasmodium in the Arthropod vector (Knell 1991)

1.2 *Plasmodium falciparum*: parasite genomics

The parasite life cycle is fairly well understood, however deeper understanding of the parasite biology has been hampered by a lack of genomic data. *P. falciparum* harbours three gnomes, two small organellar genomes, a 5.9kb mitochondrial genome, and a 35kb circular genome (Wilson *et al.,* 1996). The third genome, the nuclear genome, is approximately 30 Mb in size and is structured into 14 chromosomes, which vary in size from 0.6 to 3.5Mb in length. To address the lack of genomic data the nuclear genome has become the focus of a large scale sequencing effort by the malaria genome sequencing consortium. The consortium to date has been successful in sequencing 2 chromosomes, numbers 2 and 3 (Gardener *et al.,* 1998; Bowman *et al.,* 1999). The *P. falciparum* genome has an ~80% AT base content with one predicted gene per 4.7 kb of chromosome sequence. ~50% of the genes maintain an intro-exon gene structure characteristic of genes found in higher eukaryotes, indicating that gene splicing is an integral part of *Plasmodium* gene transcription. In addition 40 % of predicted proteins show homology matches to eukaryotic proteins (Bowman *et al.,* 1999).

The sequencing project has also identified many *Plasmodium* specific genes. Chromosomes 2 and 3 both have located at each chromosomal end, genes encoding for antigens involved in antigenic variation and cytoadhesion in *P. falciparum* malaria (Smith *et al.,* 1995; Baruch *et al.,*1995; Su *et al.,* 1995). Theses genes, named *var* genes, are members of a multi-gene family and encode the erythrocyte surface protein PfEMP-1. Research on the genes and the proteins they encode is the subject of this thesis. The sequencing effort has unearthed two previously known gene families. Members of the

rif-1 gene family are transcribed at the late trophozoite stage and are found in clusters at chromosomal ends. These genes contain a 1kb open reading frame and a two exon gene structure. *rif-1* genes contain a transmembrane sequence and recent experimental results have shown that the products of these genes are expressed at the erythrocytes surface and may be antigenically variant (Kyes *et al.,* 1999; Fernandez *et al.,* 2000). The third gene family described, *stevor* is also thought to be an antigenically variant erythrocyte surface protein family (Cheng *et al.,* 1998). The function of these gene families is unknown and requires further characterisation.

The genome sequence data are providing an invaluable resource for scientists working in malaria research. The discovery of novel *P. falciparum* specific genes and genes with homologues in other organisms will not only accelerate basic malaria research but should also facilitate the development of new drugs and vaccines.

1.3 Malaria pathogenesis

P. falciparum remains an organism of intense research not due to interest in the parasite biology *per se* but because malaria remains one of the most important infectious diseases in terms of morbidity and mortality. The symptoms of malaria vary, depending upon the species of infecting parasite, the transmission dynamics of infection and the immune status of the patient (Warrell, 1999). Malaria disease cannot be defined by a single diagnostic feature, however episodic chills, fever and sweating, interspersed by periods of wellbeing are characteristic symptoms. Classic *P. falciparum* malaria can be defined by the periodicity of febrile stages experienced by the patient, which is determined by the duration of the intra-erythrocytic cycle. Attacks of fever lasting 8-12 hours are usually precipitated by schizont rupture during blood stage parasitaemia. The symptoms of subsequent infections, after several weeks, become less marked with further infections resulting in milder symptoms in spite of persistent parasitaemia.

Blood parasitaemia bears little relationship to disease severity although hyper-parasitaemia is extremely dangerous. Immune individuals in endemic areas may be constantly parasitised, but often remain asymptomatic (Marsh, 1992). For many living in endemic areas, partial or complete immunity to malaria disease will eventually be acquired with age and symptoms will not develop in subsequent infections. However, a small proportion of patients, normally young African children, develop a severe life threatening illness when infected with *P. falciparum* malaria. Factors influencing disease severity are poorly understood but may include sporozoite dose, immune status, parasite virulence, socio-economic variables and host genetics (Weatherall, 1996).

Symptoms of severe malaria can be variable and can differ between different age groups, transmission intensities and geographical locations. Different manifestations of malaria morbidity arise from the interaction of a number of pathogenic processes including red cell destruction and toxin-mediated activation of cytokine cascades (Warrell *et al.,* 1999). However, it is believed by many that infected cell sequestration in the vascular beds of major organs plays some role in the pathogenesis of severe disease.

1.3.1 Cerebral malaria

Cerebral malaria is the best known although not the only clinical manifestation of severe malaria. It is defined as coma due to malaria (Warrell *et al.,* 1987,1992,). It is also confirmed by the histological appearance of infected erythrocytes sequestered in the micro-vasculature of some malaria post-mortem brain tissue samples (Macpherson, 1985). As has been described, during asymptomatic infection with *P. falciparum* only the uninucleate ring forms are found in the peripheral circulation of severe patients. Mature parasites, withdraw or sequester deep in the vascular beds of a variety of organs including the brain. This binding is mediated by receptors expressed at the surface of endothelial cells and ligands expressed at the surface of the parasite. Several endothelial receptors medating the adhesive phenotype have been identified (Section 1.7). The evidence suggests that a hyper-variable protein family PfEMP-1 expressed at the surface of the red cell is the parasite derived receptor mediating most of the cytoadhesion in *P. falciparum* malaria. (Newbold *et al.,* 1997 a) (Section1.6).

There seems to be a great deal of variation in receptor usage by the parasite (Newbold *et al.,* 1997 b). Heterogeneity in binding phenotype has raised the possibility that differences in either degree or site of cytoadherence may give rise to different pathological consequences. Expression of some receptors can be up-regulated in the presence of cytokines released during malaria infection (Udeinya & Akogyram, 1993). There are also differences in the parasite's ability to stimulate and control human cytokine responses (Allan *et al.,* 1993), not to mention host genetics, which undoubtedly plays a role in the development of cerebral disease (Weatherall, 1996). This has led to

the suggestion that cerebral malaria may be a consequence of particularly virulent parasites infecting a genetically susceptible host (Berendt *et al.*, 1989). To test this, attempts have been made to try and explain cerebral malaria by investigation of host cell ligand expression and the propensity of parasites associated with disease to bind to specific ligands.

Histological studies of brain endothelium have shown that the endothelial receptor CD36 is poorly expressed in brain tissue relative to other organs, and that it is in fact different in nature to CD36 expressed in other tissues (Barnwell *et al.*, 1989; Chulay & Ockenhouse 1990; Turner *et al.*, 1994). However Endothelial Adhesion Molecule 1(ICAM-1) in brain tissue is expressed at significantly higher levels in patients suffering from cerebral disease than in non-cerebral controls (Turner *et al.*, 1994).

There have been a number of studies attempting to correlate the binding to specific adhesion receptors with disease (Marsh *et al.*, 1988; Ho *et al.*, 1991; Ockenhouse, 1991 b; Chaiyaroj *et al.*, 1996; Goldring *et al.*, 1992; Ringwald *et al.*,1993; Rogerson *et al.*, 1999 a; Newbold *et al.*, 1997 b). There has been a great deal of variation in the affinity and specificity of binding of different parasite isolates to different receptors. The only consistent finding is that most parasites bind to CD36 and that CD36 binding is associated with non-cerebral disease (Ho *et al.*, 1991; Okenhouse *et al.*, 1991 b; and Newbold *et al.*, 1997 b).

Only in one study has there been any correlation between cerebral malaria and receptor binding. Newbold *et al.* (1997 b) found that ICAM-1 binding correlated with cerebral

malaria, although the correlation was not statistically significant. Drawing correlations between this type of receptor binding and symptoms of disease is fraught with complexity. The clinical definition of disease can only take into account symptoms at presentation. Some individuals presenting early with uncomplicated malaria may have gone on to develop cerebral involvement in the absence of treatment. In such correlative studies these patients would be grouped in the uncomplicated malaria group. For logistical and ethical reasons these studies are forced to draw their samples from peripheral blood and not damaged tissue. One study conducted on placental sequestration has shown that the binding specificities of parasites in the peripheral circulation differ from that of the sequestered population (Fried *et al.,* 1996). Other problems with such studies are that they rely on static assays that do not reflect the shear stress conditions under which parasites would have to bind in the blood stream. Also parasite binding to individual receptors does not necessarily reflect the natural situation where it is plausible that receptors work in co-operation to mediate cytoadherence (See section 1.7).

Host genes may also affect the progression of cerebral disease, having a direct effect on sequestration. Observations have been made suggesting an association between polymorphisms in the ICAM-1 gene and cerebral malaria. A polymorphism in the ICAM-1 gene has been found in individuals from East and West Africa, which is not present in Europeans (ICAM-Kilifi). In a large case control study it was found that homozygotes for this allele had a significantly higher risk of developing cerebral malaria than other genotypes (Fernandes-Reyes, *et al.,* 1997).

Elevated cytokine production may also play a role in the sequestration process. High plasma levels of TNF-α have been associated with cerebral malaria (Kwiatkowski *et al.*, 1990). This cytokine has been proposed to affect disease via its local effect on endothelial receptor expression (Berendt *et al.*, 1989). There is evidence that TNF-α production is up-regulated in both *P. vivax* and *P. falciparum* but the ensuing pathology differs between the two (reviewed by Kwiatkowski 1995). TNF-α and other cytokines may increase the levels of sequestration locally by activating endothelial cells, causing the up-regulation of host derived cytoadherence receptors (Rothlein *et al.*, 1988; Pober *et al.*, 1988). This up-regulation by cytokines has been demonstrated *in vitro* for the parasite cytoadherence receptor ICAM-1 (Berendt *et al.*, 1989, 1992)

1.3.2 The rosetting phenotype in infected red blood cells

Another adhesive phenotype, which some authors have associated with severe disease is that of rosetting, the ability of infected erythrocytes to adhere to uninfected erythrocytes. The rosetting phenotype was first described in *P. fragile* which sequesters in its natural host the toque monkey (David *et al.,* 1988). This phenomenon has also been observed in *P. falciparum*, which also sequesters (Carlson *et al.,* 1992). These initial experiments revealed that rosettes could be disrupted by immune serum in a specific manner, suggesting that the molecules involved were antigenic (David *et al.,* 1988, Carlson *et al.,* 1992). However this study shed no light on whether this phenomenon occurred *in vivo* or was clinically relevant.

Association of rosetting with severe disease was first investigated in The Gambia where it was found that all patients suffering from cerebral malaria possessed parasites that formed rosettes, a phenotype exhibited by only 18% of the uncomplicated malaria cases (Carlson *et al.,* 1990). The ability of immune serum to inhibit or reverse rosettes was also analysed. Antisera from cerebral cases failed to reverse rosetting while almost 40% of sera from uncomplicated malaria cases did reverse rosetting. Data from *ex vivo* studies show that rosetting enhances micro-vascular obstruction by compounding the effects of cytoadhesion. Parasites capable of cytoadherence and rosetting showed a 3.6 fold increase in flow resistance compared to a 2.7 fold increase in parasites which only cytoadhere (Kaul *et al.,* 1991). Heparin and antibody can disrupt these clusters, the latter

observation supporting the contention that antibody which inhibits rosetting *in vitro* may also play a role in preventing or reversing sequestration *in vivo*. Although there is a strong link between this phenotype and the pathogenesis of severe disease the ultimate significance of this phenotype remains unclear. Hypotheses to explain its selective value to the parasite have included the idea that phagocytosis is prevented through additional protection from host antibody, and that rosetting is a mechanism to promote red cell invasion. The latter property has been tested and it was found that there was no significant difference in invasion rates between rosetting and non-rosetting parasites (Clough *et al.*, 1998).

1.4 Placental malaria and *P. falciparum* cytoadhesion

The brain is only one of several organs within which the mature infected erythrocyte sequesters. In pregnant women, especially those experiencing their first pregnancy (primigravidae), the placenta represents another site (Fried *et al.,* 1996). This has clinical consequences, as these women become more susceptible to frequent and severe forms of malaria infection (Gilles *et al.,* 1969; Brabin *et al.,* 1983). Pregnant women suffer two- to ten-fold higher mortality due to severe malaria than non-pregnant women (Brabin, 1983, 1991). Abortion, still birth, premature delivery and low birth weight of the infant are also common. The most frequent presentation of maternal malaria is anaemia (Brabin, 1983), and the most common effect this has on the foetus is a significant reduction in birth weight (Bruce-Chawatt, 1952; Gilles, 1969; McGregor, 1983, Watkinson & Rushton, 1983; Cot *et al.,* 1992; Matteelli *et al.,* 1996; Steketee *et al.,* 1996).

Malaria can cause low birth weight via different aetiologies including intra-uterine growth retardation (IUGR), premature delivery or both. However, a consensus has emerged that IUGR is the predominant mechanism (McGregor 1984; Steketee, 1996) and that it is associated with placental infection. Malaria-induced low birth weight is thus associated with a significantly increased risk of mortality and morbidity in the first year of life.(Bloland, 1996). Parasitised erythrocytes have consistently been described in the intervillous spaces in placentae delivered at term. The predominant stage found is the trophozoite, although schizonts have also been reported (Rogerson & Beeson 1999). Malaria pigment is however present in several sites during the early stages of infection, concentrating in the macrophages, intervillous spaces, trophoblasts, Hoffbauer cells and

in fibrin deposits in the villous stroma. Pigment disappears after active infection is resolved (Bulmer *et al.,* 1993). This clearance occurs within months and thus any pigment present in term placentae probably derives from infections acquired during the second half of pregnancy. The appearance of parasite pigment is not the only histological change observed in placental infection. Monocytes sequester in the intervillous spaces, cytotrophoblastic cells become more common and there is a thickening of the trophoblastic basement membrane (Galbraith *et al.,* 1980; Walter *et al.,* 1981; Yamada *et al.,* 1989)

1.4.1 Ligand receptor interactions associated with placental malaria infection

Histological examination of placenta may show signs of acute infection (the presence of infected erythrocytes in the intervillous spaces), past infection (malarial pigment in leukocytes) or both. Placental parasitaemias exceed 50% in contrast to peripheral parasitaemias, which are typically between 0.5 and 2.0% (Bulmer *et al.,* 1993). Bulmer *et al.* (1993) and McGregor, (1984) postulated that the accumulation of parasites in the placenta results from sequestration, shielding the parasites from immune destruction. This observation has been linked with the ability of the infected erythrocyte to adhere *in vitro*. Mature infected erythrocytes exhibit a number of adhesive specificities *in vitro*. It has been shown that the glycosaminoglycan chondroitin sulphate A (CSA) is an infected erythrocyte adhesive receptor (Rogerson *et al.,* 1995). CSA binding is not a property of all parasites. Among field parasites only a minority bound to CSA *in vitro* (Chaiyaroj *et al.,* 1996).

The identification of CSA as *P. falciparum* cytoadhesion receptor prompted a more systematic search for its biological significance. Fried *et al.*(1996) investigated the placenta as a possible site for *in vivo* cytoadherence via CSA, as CSA is expressed on the syncytiotrophoblast of the placenta. Parasites sequestered in the placenta adhered to trophoblastic villi, extracellular villi and syncytial bridges in a CSA dependent manner. CSA inhibits and reverses binding of infected erythrocytes to placenta. Pre-treatment of tissue samples with chondroitinase ABC, an enzyme which hydrolyses CSA, also inhibited adhesion. Parasites taken from the peripheral circulation failed to adhere in substantial numbers to CSA but bound to another cytoadhesion receptor, CD36. Parasites isolated from the placenta were unable to bind to CD36 or ICAM-1 but bound in significant numbers to CSA. Fried *et al.* (1996) suggest that *P. falciparum* infection consists of a mixed population (in terms of the adhesive phenotype they express) and that parasites expressing the ligand for adhesion to CSA are selectively sequestered in the placentae of pregnant women. The authors also suggest that this phenomenon is more common in primigravid women due to the development of anti-adhesive antibodies with successive pregnancies. Multigravid women possess antibodies which are able to block adhesion to CSA and these antibodies are absent from men, children and primigravid women (Fried *et al.*, 1998; Ricke *et al.*, 2000). The presence of these antibodies also correlate with reduced placental infection. This antibody response is strain-independent (Fried *et al.*, 1998).

The issue of placental sequestration has been complicated by the recent identification of hyaluronic acid (HA) as a cytoadhesion receptor for the infected erythrocyte, which is

also expressed in placental tissue. In a recent study it has been shown that 80% of parasites isolated from the placenta adhere to hyaluronic acid *in vitro*, compared to 36% of parasites isolated from the peripheral circulation of pregnant women and 33% of parasites isolated from the peripheral circulation of children (Beeson *et al.*, 2000). Laboratory isolates selected for binding to CSA also adhere via HA. However, this cross-binding is not consistently found in field isolates. The authors thus suggest that the binding epitope for these two adhesive phenotypes may be expressed on the same protein, but this is yet to be proven. It is difficult to say whether these binding receptors work synergistically in placental sequestration or whether they merely provide the parasite with a number of options for binding to the placenta. More research into this area will provide answers to these questions.

1.4.2 Host factors which may also influence placental infection

Host factors may exacerbate the pathological effect of parasites sequestering in the placenta. Placental intervillous spaces may be infiltrated to varying degrees by leukocytes, predominantly mononuclear cells which often contain pigment (Galbraith *et al.*, 1980; Bulmer *et al.*, 1993). There is a higher proportion of leukocytes in intervillous blood than peripheral blood. In over half of the moderately to heavily parasitised placentae, mononuclear cells represented greater than 5% of intervillous cells. The degree of infiltration can be intense, resulting in chronic intervillousitis (Ordi *et al.*, 1998). This condition occurs in approximately 10% of placentae isolated and most

frequently in primigravidae. Intervillousitis also appears to be strongly associated with low birth weight (Ordi *et al.,* 1998).

Many cells within the placenta including Hoffbauer cells, cells of the syncytiotrophoblast, uterine epithelial cells, amniotic membranes, foetal endothelium, and intervillous maternal leukocytes can produce a wide variety of cytokines and chemokines (Robertson *et al.,* 1994). Although the Th1-Th2 dichotomy has not been firmly established in human pregnancies, the pro-inflammatory Th1 and the anti-inflammatory Th2 response may help explain the immune response to malaria in the placenta. Robertson *et al.,* (1994) suggest that successful pregnancy and foetal growth are associated with a predominant Th2 response in the placenta, whereas a predominant Th1 response may lead to foetal loss. Recently a study was conducted on the cytokine responses in the placenta in relation to malaria infection, which suggested that infection induces a shift towards the production of a Th1 response, resulting in adverse pregnancy outcome (Fried *et al.,* 1998).

1.5 Immunity to malaria and antibody responses against PfEMP-1

Understanding of the pathogenesis of malaria has provided an insight into the mechanisms of pathology. This understanding combined with an understanding of the immune response against the parasite may permit the development of public health interventions such as vaccines. In endemic areas a degree of immunity only appears to develop after multiple infections over several years. This immunity appears to develop in two phases. An anti-disease immunity, signified by a reduction in clinical symptoms, followed by an anti-parasite immunity, signified by a reduction in parasitaemia. Children from the age of six months to five years are at risk from severe malaria and even in adults this immunity is rarely complete (Marsh *et al.,* 1992). The persistence of the parasite in immune individuals is thought to result from immune evasion mechanisms such as antigenic variation (Roberts *et al.,* 1992), the ability to down-regulate host immune responses (Urban *et al.,* 1999) and possibly to antagonise cytotoxic T-lymphocyte responses (Gilbert *et al.,* 1998).

1.5.1 The species specificity of immune responses to malaria

Evidence of the species specificity of immunity to malaria comes from analysis of neurospyhillitic patients treated with malaria as a therapeutic measure. These treatments used several different *Plasmodium* species, with individual patients often receiving

multiple inoculations during the course of treatment. This work demonstrated that some degree of immunity was observed in individuals infected with homologous species of *Plasmodium*, an immunity which was not protective against inoculations with heterologous species (Covell & Nicol, 1951; Jeffrey, 1966). These conclusions have recently been challenged by epidemiological data suggesting that in areas where *Plasmodium falciparum* and *Plasmodium vivax* coexist there is a dynamic relationship between them. The authors observed an apparent suppression of *P.vivax* infection by *P. falciparum* and suggest that the nature of this suppression could be based on a level of immunological cross-protection (Maitland *et al.*, 1996).

1.5.2 Strain Specific immunity to *P. falciparum*

Analysis of malaria treatment of neurosyphillitic patients also revealed that there are significant differences in clinical symptoms between patients infected with heterologous strains of the same species as compared to those infected with homologous strains. Any acquired immunity is essentially strain-specific (Covell & Nicol 1951; Jeffrey, 1966). These results were confirmed in the primate model *P. knowlesi* (Brown & Brown, 1965, Brown *et al*, 1970), studies which also led to the identification of variant-specific immune responses (section 1.6).

1.5.3 The stage specificity of the immune response to *P. falciparum*

1.5.3.1 liver stage immunity

Immunity against *P falciparum* is essentially stage-specific. Almost all of the information available on the protective immune response against the sporozoite and hepatic stages of infection is based on data generated from immunisation experiments with irradiated sporozoites (Orjih *et al,*. 1980). Infection with *P. berghei* sporozoites can be immunogenic and elicit a response which protects against sporozoite challenge (Beaudoin *et al.,* 1977). It is essential for the induction of this immune response that the sporozoites are able to invade and at least partially develop in the hepatocytes (Herrington *et al.,* 1991). The predominantly expressed protein at this stage of the parasite's life cycle is the circumsporozoite protein (CS). The gene determining this protein was first cloned in 1984. The gene sequence consists of a repeat region flanked by two repetitive conserved regions (Enea *et al.,* 1984). The protein is thought to be involved in sporozoite mobility and is essential for binding to and invasion of the hepatocyte (Stewart, 1986; Cerami *et al.,* 1992). Human volunteers immunised with irradiated sporozoites produce CD8+ cytotoxic T-cells with specific activity against CS protein (Kumar *et al.,* 1988), a response subsequently observed in adult Kenyans (Sedegah *et al.,* 1992) and Australians who had lived in malaria endemic areas (Doolan *et al.,* 1991). Recently its has been demonstrated that HLABw53 restricted CTLs recognise a polymorphic epitope on the CS protein which antagonises the CTL response in-vitro (Gilbert *et al.,* 1998). This CTL response may be important in clinical immunity given that the HLA allele Bw53 has been associated with protection from severe disease (Hill *et al.,* 1992).

1.5.3.2. Blood stage immunity

The next stages in the parasite life-cycle to be exposed to the host immune response are the asexual blood stages, which are responsible for the mortality, pathology and morbidity associated with malaria disease. Protective immunity to these stages is thought to be both antibody and cell-mediated, but again the precise mechanisms of protection are unclear. In some murine malarias such as *P. chabaudi*, control and elimination of the parasite can be achieved in an antibody independent manner (McDonald & Phillips, 1980; Langhorne *et al.,* 1989; Taylor-Robinson, 1993). For *P. chabaudi chabaudi* AS strain the control of the primary acute parasitaemia is independent of antibody but the subsequent elimination of the parasite is dependent on antibody. For the latter parasite, CD4+ T-cells are essential for mice to survive infection (Langhorne *et al.,* 1989). In this murine model the primary acute parasitaemia seems to be mediated by CD4+ Th1 cells (the subset of CD4+ cells associated with antibody-independent immunity), whereas immediately following the decline in parasitaemia there is a switch to the CD4+ Th2 response (associated with antibody production) (Langhorne *et al.,* 1989; Taylor-Robinson & Phillips, 1994 a, 1994 b). These animal experiments now have support from epidemiological findings, which have shown that the reduction of CD4+ cells brought about by HIV infection contributes to and increased risk of developing clinical disease (Whitworth *et al.,* 2000)

The merozoite stage of infection is also a target for the host immune response, a response that has been correlated with clinical immunity (Egan *et al.,* 1996). The most abundant protein on the surface of the merozoite is the merozoite surface protein -1

(MSP-1). This protein consists of a complex of polypeptides, which are proteolytically cleaved from the surface of the merozoite prior to merozoite invasion. The protein is anchored to the surface of the merozoite via a GPI anchor and can be divided into seventeen variable semi-variable and conserved sequence blocks (reviewed by Cooper *et al.,* 1993). The protein is subject to several cleavage events prior to invasion, the penultimate cleavage leaving a 42 Kd protein product attached to the merozoite surface. The ultimate cleavage step can be blocked by monoclonal antibody, which also has the ability to block invasion *in vitro* (Blackman *et al.,* 1994). In primate and mouse models, immunisations with recombinant protein or by passive transfer of antibody confer protection against homologous challenge. This supports epidemiological evidence which suggests that the development of antibody responses to MSP-1 correlates with protection from both clinical malaria and parasitaemia (Egan *et al.,* 1996).

1.5.3.3 Immune responses to the *P. falciparum*-infected erythrocyte surface

The merozoite, once it has successfully invaded the red cell, develops into a trophozoite. The red cells express no MHC-antigens and thus cannot present parasite proteins to the host immune system. It has been suggested that the parasite's metabolites are exported to the surface of the infected red cells via proteins expressed through knob structures present at the red cell surface (Berendt *et al.,* 1994). Knob proteins appear at the surface of the infected red cell. They are a complex of proteins, which include the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). This protein family is involved in cytoadherence, antigenic variation and is the target of clinical immunity to *P. falciparum*

(Reeder & Brown 1996). As far back as 1938 it was show by agglutination assays that red cells containing mature trophozoite stage parasites could be agglutinated by immune serum, from which it was concluded that the surface of the infected red cell was of immunological importance (Eaton *et al.,* 1938).

In 1986, Marsh & Howard published experiments to examine the presence of these variant-specific agglutination responses in individuals living in endemic areas and to see whether these responses correlated with protection from severe disease (clinical immunity). They conducted agglutination experiments using acute sera, obtained on the day of reported infection and convalescent sera, collected 21 days post infection from the same patient. In most cases, acute sera from children aged one to five years did not recognise their infecting parasite whereas the convalescent sera of each child did. Agglutination of heterologous parasites by the children's acute or convalescent sera was relatively rare. However, sera collected from adults from the same area possessed agglutinating antibodies capable of agglutinating a wide range of parasite isolates (pan-agglutinating antibodies). From these experiments they concluded that the agglutinating antibody response was isolate specific and with age one could develop an agglutinating response against a number of isolates either through exposure to the entire antigenic repertoire of the parasite or through exposure to conserved determinants on the red cell surface.

Absorption and elution experiments have shown that adult sera absorbed onto and eluted from the surface of the infected red cell are able to pan-agglutinate a number of different parasite isolates whereas the remaining non-absorbed sera lose all ability to pan-

agglutinate (Marsh & Howard, 1986). The authors inferred from this result that the pan-agglutinating antibody response is to conserved determinants. Agglutination studies conducted in a variety of geographical locations have confirmed that, at least in children, the agglutination response is isolate specific (Forsyth *et al.,* 1989; Reeder *et al.,* 1994; Bull *et al.,* 1998). Many studies indicate that many if not all adults living in malaria endemic areas acquire pan-agglutinating antibodies (Marsh & Howard, 1986; Forsyth *et al.,* 1989; Aguire *et al.,* 1992). However, Reeder *et al.* (1994) were unable to isolate these pan-agglutinating antibodies. The existence of pan-agglutinating antibodies and how they are derived remains contentious; however, the answers to these questions are pertinent to the development of a malaria vaccine against this stage of infection.

Although a number of studies have confirmed that increased agglutination response is directly proportional to the age of the serum donor (an indirect measure of clinical immunity) only two studies have directly correlated the agglutinating response with protection against clinical malaria (Marsh *et al.,* 1989; Bull *et al.,* 1998). Bull *et al.,* conducted a large prospective study of the agglutinating antibody response and its relationship with clinical immunity. They showed that children who develop clinical malaria show a significant reduction in the ability of their pre-infection sera to agglutinate the infecting parasite, compared to age-matched asymptomatic controls. They thus concluded that the agglutinating antibody response correlates to some degree with protection. They also showed that there was an almost exclusive appearance during clinical disease of parasite variants corresponded to "gaps" in the child's developing repertoire of PfEMP-1 antibodies. The authors inferred from this that pre-existing

antibodies only provide protection against the variants to which they are directed (they are isolate specific).

Another study looking at the agglutinating antibody response, in an area of meso-endemic malaria in Sudan (Giha *et al.,* 1998), has demonstrated that the pan-agglutinating response in the post-transmission season is greater than the pre-transmission response, indicating that this response is a) against conserved determinants and short-lived, b) against conserved determinants and boosted by infection or c) against greatly diverse antigenic determinants. They also show that the acquisition of pan-agglutinating antibody can occur in the absence of clinical disease, an observation that has potential implications for the treatment of sub-clinical infections. Further to this Bull *et al.* (1999) have observed that the most frequently agglutinated isolates (that is, the variant specific antigen recognised by most serum) tended to be from individuals suffering from severe disease. This suggests that clinical immunity is acquired through immune responses against specific antigenic variants, which are common or at least commonly recognised by the host immune system.

A number of studies have shown that a natural acquired immune response against the trophozoite stage of malaria prevents or reverses binding of infected erythrocytes to cell lines (Forsyth *et al.,* 1989) and purified receptors (Reeder *et al.,* 1994) *in vitro*. Indeed a high level of agglutinating antibodies seems to correlate with having a high degree of anti-binding antibody (Forsyth *et al.,* 1989). One study has indicated that individuals who presented with severe disease tended to lack antibodies, which inhibited or reversed rosetting. A number of individuals who presented with mild disease in the same study

possessed anti-rosetting antibodies, the implication being that this immune response protects individuals against severe disease (Carlson *et al.,* 1990). Recently evidence also suggests that the immune response against the infected erythrocyte's surface protects multigravid women from the adverse effects of malaria during pregnancy (Ricke *et al.,* 2000). This has been discussed in greater detail in section 1.4.

1.6 Antigenic variation and the identification of PfEMP-1

It is known that some *Plasmodium* species maintain chronic erythrocytic infections over long periods of time (Brown & Brown 1965). One possible biological phenomenon that could explain this observation is that the parasite modifies its antigenic surface during infection to escape the developing immune response. Clonal antigenic variation has been observed in other parasitic protozoa such as *Trypanosoma brucei* and *Giardia lamblia* and could explain, in part, chronic malaria infection and the slow development of immunity.

The first evidence suggesting antigenic variation in malaria was provided by serological experiments employing a Schizont Infected Cell Agglutination assay (SICA) developed by Eaton *et al.* (1938) in which immune serum from monkeys immunised with *Plasmodium knowlesi* agglutinated the mature trophozoite forms of the parasite. Brown & Brown (1965) demonstrated that the target of this agglutinating response was a novel antigenically variant antigen expressed at the infected erythrocytes' surface. *P. knowlesi* schizonts were agglutinated by convalescent sera obtained from the infected monkey two to three weeks post inoculation. Serum immunoglobulin reacted with an antigen being expressed at the parasites surface. However the inoculation of parasites into a monkey already possessing antibodies to the specific variant being expressed resulted in the emergence of a parasite population expressing a novel antigenic type. The authors

concluded from these experiments that *P. knowlesi* like *T. brucei,* was capable of clonal antigenic variation.

Other authors suggested that the emergence of an antigenically distinct parasite population might represent immune selection of a parasite population from a genetically mixed parasite pool. Thus these experiments were eventually repeated with cloned parasites and similar results obtained (Barnwell *et al.,* 1983). It was also shown that the target of the agglutinating antibody response against the variant antigen seemed to be modulated by the spleen, which the authors correlated with virulence (Barnwell *et al.,* 1983). Experiments in squirrel monkeys demonstrated that antigenic variation and splenic regulation of the variant antigen were also properties of *P. falciparum.* These experiments were the first to link changes in variant antigen expression with changes in the cytoadhesion properties of *P. falciparum* (Hommel *et al.,* 1983) (see section 1.7).

As similar experiments were conducted on other species of *Plasmodium* it became apparent that antigenic variation was not a phenomenon specific to just a few species of *Plasmodium* but probably an immune evasion strategy employed by all species . In *P. fragile* there is also some evidence that switching of the variant antigen occurs in sequential order (Handunnetti *et al.,* 1987).

Parallel to these *in vivo* studies, biochemical experiments on *P. knowlesi* were being performed with the aim of defining the SICA antigen involved in antigenic variation. It was shown that this protein could be detected by radio-iodination and was approximately 210 kDa in size. It could also be immuno-precipitated specifically by

antibody which agglutinated the same parasite in a variant specific manner (Barnwell *et al.*, 1983). Similar experiments conducted on *P. falciparum* identified the *P. falciparum* equivalent of SICA which was termed *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) (Leech *et al.*, 1984). Cloned parasites cultured *in vitro* over a number of months eventually gave rise to antigenically different parasite populations, which expressed different PfEMP-1 molecules of different size and antigenicity and again could only be immuno-precipitated by serum that agglutinated the parasites from which these proteins were derived. This was the first evidence that antigenic variation also occurs *in vitro* (Biggs *et al.*, 1991).

In a set of experiments conducted by Roberts *et al.* (1992) 7 sub clones were produced from the ITO-4 parasite line. Twenty-one clones were produced during a two month cloning period. During this time the antigenicity of some of the cloned parasites had switched as defined by a change in agglutination and cytoadherence phenotype. From these data it was calculated that antigenic variation occurred at a rate of 2% per parasite generation. Biggs *et al.* (1992) demonstrated that cloning followed by selection for adherence of infected erythrocytes to an endothelial cell line resulted in the selection of specific PfEMP-1 variants of similar size and antigenicity. The size of the PfEMP-1 variant and its antigenicity were shown to be distinct from the parental unselected clone. These experiments showed that PfEMP-1 is antigenically variant, and is linked to cytoadherence. However, in the absence of data indicating the existence of a multi-gene family not all malariologists were convinced.

1.6.1 *Var* genes

In 1995, using a variety of methods, the multi-gene family encoding PfEMP-1 was finally identified (Su *et al.*, 1995 Smith *et al.*, 1995, Baruch *et al.*, 1995). Baruch *et al.*, (1995) conducted an elegant set of experiments using agglutinating serum recognising the Malayan Camp parasite strain to identify a clone expressing PfEMP-1 epitopes expressed in a λgt11 cDNA expression library. Su *et al.* (1995) fortuitously identified a multi-gene family with PfEMP-1 type characteristics while in search of genes determining chloroquine resistance on chromosome 7. Through a collaborative effort it was show that the sequences identified matched the criteria required for PfEMP-1 type proteins. Baruch *et al.* (1995) were able to show that antisera raised against the sequence expressed in a heterologous expression vector, recognised the surface of the infected red cell. The third paper in the set representing this major discovery demonstrated that transcription of a specific gene family member correlated with the expression of a corresponding PfEMP-1 specificity (Smith *et al.*, 1995). The gene family was named *var* and is thought to be present as approximately 50 copies per *P. falciparum* genome (Su *et al.*, 1995). The genes vary in size between 4 and 13 kb, and always consist of two exons separated by an approximately 1 kb intron, which is adjacent to the trans-membrane region of the gene. Exon-1 encodes the part of the protein which lies extracellular to the red cell membrane and is divided up into 1-2 Cysteine-Rich Interdomain Regions (CIDR) and 2-7 Duffy binding ligands (DBL) domains. one CIDR and four different types of DBL domain groups were originally identified, DBL-1 to DBL-4, based on specific conserved sequence motifs previously noted in the *P. vixax* duffy binding protein (Su *et al.*, 1995). However, recently a second

type of CIDR domain and another DBL homology group have been defined. The nomenclature has also changed, replacing the original numerical labelling of CIDR and DBL domains with the Greek alphabet (i.e. DBLα, β, γ, δ, and ε) (Smith *et al.,* 1998, 2000 b). The authors also discovered a new C2 domain, which is always found 3' of the DBL-β domain. A schematic representation of a *var* gene is shown in Figure 1.4

Var genes have been localised to the subtelomeric region of each of the 14 plasmodial chromosomes and to the internal regions of a number of them (Rubio *et al.,* 1996). The *var* gene sequence exhibits a great deal of diversity both within and between clones from different geographical locations (Kyes *et al.,* 1997; Ward *et al.,* 1999). This may contribute to antigenic diversity with regard to the immune response against this stage of the parasite. Analysis of parasite clones which undergo meiosis under laboratory conditions have revealed that this diversity is generated at a rate far greater than would be expected by homologous recombination (Freitas-Junior *et al.,* 2000). The authors suggest that in addition to homologous crossover events, *var* gene diversity in *P. falciparum* is generated by ectopic gene conversion events between heterologous chromosomes.

Figure 1.2 Var gene schematic

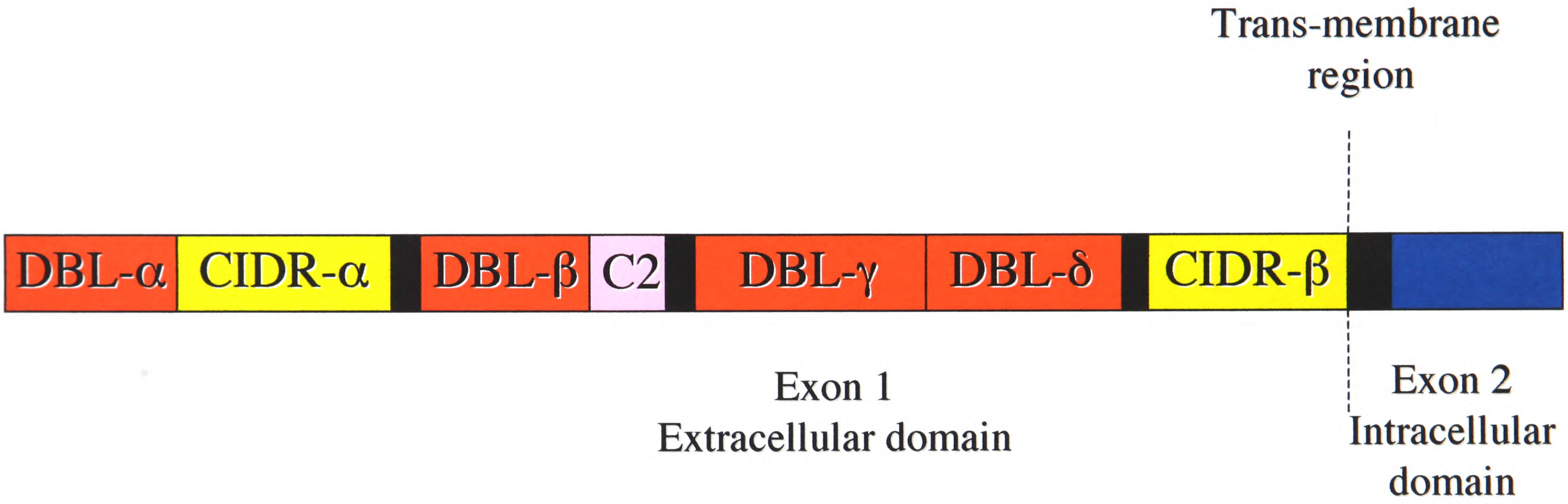

Figure 1.4. Schematic of *var* gene. All *var* genes possess a 2 exon gene structure, the second exon (Blue) encoding an intracellular domain. The extra cellular portion can have between 2-7 DBL- domains (Red) and a C2 domain (Pink) which is always found 3' of the BDL-β domain. There are between 1-2 CIDR domains (Yellow). *Var* genes range in size between 4-13 kb and are located on all *P. falciparum* chromosomes.

1.6.2 *Var* gene transcription

Var genes are known to be transcribed from both internal and subtelomeric chromosomal locations (Fischer *et al.,* 1997), thus differentiating their expression from that of the African trypanosome vsg (variant surface glycoprotein) genes whose expression is largely limited to telomeric expression sites (Borst *et al.,* 1995). It seems likely that in *P. falciparum* most, maybe all, *var* genes can be transcribed, regardless of their chromosomal location. Early developmental stage parasites (rings or early trophozoites) are capable of simultaneously expressing several *var* genes from different chromosomes (Fischer *et al.,* 1997; Scherf *et al.,* 1998; Chen *et al.,* 1998 b). However, the expression of *var* genes in mature parasites is thought to be less heterologous and may even be monogenic (Scherf *et al.,* 1998; Chen *et al.,* 1998 b).

Although many mechanisms of transcriptional regulation of *var* genes have been postulated (Borst *et al.,* 1995) it is becoming clear that *var* gene expression could have some element of post transcriptional regulation. Chen *et al.,* (1998 b) and Scherf *et al.,* (1998) suggest that early developmental forms may undergo a process of 'loose transcription' during early ring stage, resulting in the short-lived activation of many and perhaps all *var* genes. By the trophozoite stage of infection, transcripts of a single *var* gene may in some way be selected for expression on the cell surface. These findings have not been supported by all workers in the field, some of whom suggest that only one full length *var* transcript is present at ring stage (Taylor *et al.,* 2000).

1.7 The molecular basis of cytoadherence

P. falciparum is the most pathogenic of the human malaria species. A feature which distinguishes *P. falciparum* infection from that of the other malaria species infectious to humans, *P. ovale, P malariae* and *P. vivax,* is the absence of mature trophozoites/schizont forms in the peripheral circulation. Post-mortem samples of specific organs such as the brain frequently show that capillaries are occluded by these mature parasite forms, a process known as sequestration (MacPherson *et al.,* 1985). Sequestration may be due to a variety of cellular interactions as the parasite is able to bind *in vitro* to a number of cell types. These include monocytes, macrophages, platelets, lymphocytes, uninfected erythrocytes, infected erythrocytes and syncytiotrophoblast surfaces of placental villi. The relative importance of these interactions *in vitro* are unknown, but sequestration clearly has important consequences for both host and parasite.

1.7.1 The host receptors for *P. falciparum* cytoadherence and sequestration

Due to the relative wealth of information on receptors expressed by the host, significant progress has been made in our understanding of this half of the host-parasite interaction. *In vitro* models of endothelial binding first demonstrated that the most plausible explanation for infected cell sequestration was that the infected erythrocyte adhered to microvascular endothelium and that the presence of parasites in the micro-vasculature

was not simply due to mechanical obstruction as has been suggested by some authors. Human umbilical vein endothelial cells (HUVEC), amniotic endothelial cells and C32 melanoma cells all mediate cytoadherence *in vitro* and each were pivotal in identifying cytoadherence receptors. An antibody against the human cell surface antigen OKM-5 (which was later identified as the leukocyte differentiation antigen CD36) inhibited adhesion of infected erythrocytes to C32 melanoma cells (Barnwell *et al.,* 1989). A second adhesion receptor was identified by demonstrating that parasitised erythrocytes could adhere to petri dishes coated with thrombrospondin, another glycoprotein involved in adhesion migration, proliferation and differentiation of leukocytes (Roberts, *et al.,* 1985). In an elegant set of experiments Berendt *et al.,* (1989) demonstrated that transfection of COS cells with cDNA from the ICAM-1 encoding sequence permitted these cells to support the cytoadhesion of infected erythrocytes. ICAM-1 is a member of the immunoglobulin superfamily which plays a major role in the adhesion and transmigration of leukocytes from the blood to the tissues during inflammation. COS cell transfection was subsequently used to provide definitive evidence that another cell surface molecule, CD36 mediates cytoadherence (Oquendo *et al.,*1989). Other endothelial receptors responsible for recruitment of leukocytes during inflammation also appear to be involved in cytoadherence. Two of these receptors, endothelial cell adhesion molecule-1 (ECAM-1) and vascular cell adhesion molecule –1 (VCAM-1) mediated cytoadhesion after cytokine induction of their expression at the surface of HUVEC cells (Ockenhouse *et al.,* 1991b). The list of cytoadherence receptors now includes, P-Selectin (Ho *et al.,* 1998), PECAM-1 (Treutiger *et al.,* 1997), hyaluronic acid (Beeson *et al.,* 2000) and chondroitin sulphate-A (Rogerson *et al.,* 1995), indicating that the parasite exhibits an array of host ligands which it may use to attach itself to host

endothelium. As the list has grown ever longer some authors have suggested that only one or two of these receptors may be of clinical importance. This idea has been investigated, but the results are still inconclusive (see section 1.3).

1.7.2 Are static assays of adhesion good models of *in vivo* parasite mediated sequestration?

Most studies of cytoadherence of parasitised erythrocytes have employed static assays because they are technically simple and inexpensive. However, experiments conducted in a static environment ignore the shear forces exerted on cell-cell interactions by circulating blood. These forces presumably affect the ability of parasitised erythrocytes to localise in the venular circulation *in vivo*. Flow based assays are therefore likely to be better able to model the dynamic relationship which occurs between parasitised erythrocytes and the lining of the endothelium. Animal models of infection such as *P. falciparum* infection of *Aotus* or *Saimiri* monkeys has provided some insight into cytoadherence under flow, but extrapolation from these systems to that of the human disease must be interpreted with caution because there are many differences in malaria induced pathology in these animal models (Miller *et al.,* 1969; Whiteley *et al.,* 1987). *Ex vivo* experiments using rat mesoappendix have yielded some interesting results. Increase in resistance to flow correlates with the occurrence of parasitised erythrocyte sequestration, but defining the precise interplay of cytoadherence receptors in these systems is difficult (Raventos-Suarez *et al.,* 1985).

In vitro flow based assays permit assessment of both qualitative and quantitative aspects of the adhesive interaction. When infected erythrocytes are passed over either endothelial cell lines or purified proteins the different endothelial cell expressed receptors operate with different efficiencies and mediate characteristically different types of adhesion. Experiments investigating the effect of flow on adhesion to ICAM-1, TSP and CD36 showed that ICAM-1 was least sensitive to increased sheer stress and that TSP adhesion appeared to be transient. CD36 mediated static adhesion whereas infected erythrocytes appeared to roll continuously when attached to ICAM-1. The velocity of this rolling appeared to be inversely related to the concentration of ICAM-1 (Cooke *et al.*, 1994). The observation that CD36 is more sensitive to flow and that increasing ICAM-1 density reduces the velocity of rolling is consistent with the idea that ICAM-1 traps infected erythrocytes and slows them down prior to their attachment to CD36 in a static fashion. This idea has recently been tested. It has been demonstrated that up-regulation of ICAM-1 on human dermal microvascular endothelial cells (HDMEC) (which also expresses CD36) dramatically increases adhesion via this cell line and that these two receptors work synergistically *in vitro* to support cytoadhesion (McCormick *et al.*, 1997).

1.7.3 Parasite derived proteins that mediate cytoadhesion

The *P. falciparum* parasite derived ligands which mediate cytoadherence have not been easy to identify. When *in vitro* cytoadhesion was first described in 1981, electron microscopic examination of this interaction revealed that cytoadhesion appeared to be

mediated by protein protrusions, subsequently named knobs, present at the infected erythrocyte surface (Udeinya *et al.,* 1981). The knob proteins appeared to be a complex of proteins, but erythrocyte surface expression of these proteins is know known to be dependent upon the expression of the knob associated histidine-rich protein, KAHRP (Kilejian *et al.,* 1979; Crabb *et al.,* 1997). The selection of knobless clones still capable of cytoadherence led to the suggestion that knobs may be involved in but are not essential for cytoadhesion (Biggs *et al.,* 1990; Udomsangpetch *et al.,* 1989). Transfection studies, subsequently demonstrated that knobs were indeed not essential for static adhesion but play a functional role in adhesion under flow conditions (Crabb *et al.,* 1997).

1.7.3.1 The role of PfEMP-1 in cytoadhesion

Experiments attempting to identify the protein mediating antigenic variation in malaria were pivotal in defining the parasite-derived cytoadhesion receptor. As discussed in section 1.6, experiments on *P. knowlesi* demonstrated that expression of the variant antigen at the surface of the infected erythrocyte was mediated by the spleen (Barnwell *et al.,* 1983). These experiments were also conducted in animal models which also sequester, revealing that removal of the spleen not only modulates expression of the variant antigen but also the ability of parasites to sequester *in vivo* (Handunnetti *et al.,* 1987; Hommel *et al,.*1983). Removal of the spleen resulted in the appearance of mature stages of parasites in the peripheral circulation. Infection of intact animals resulted in both the withdrawal of mature forms from the peripheral circulation and the expression of the variant antigen. The implication of this was that agglutination and sequestration were mediated by the same molecule. This suggestion was supported by the observation

that sera capable of agglutinating field isolates could also inhibit cytoadherence *in vitro* (Forsyth *et al.,* 1989, Reeder *et al.,* 1994).

The variant antigen in *P. falciparum* was biochemically defined and termed PfEMP-1. This was followed some years later by the coning of the gene family encoding these proteins (Section 1.6). The cloning of the *var* gene family permitted heterologous expression of the binding epitopes thought to mediate cytoadherence. Molecular studies demonstrated that a semi-conserved region within the CIDR-α domain of a specific *var* gene was capable of binding to CD36, and inhibited CD36 mediated cytoadherence (Baruch *et al.,* 1997). Expression of this domain in COS cells supported cytoadhesion of infected erythrocytes to the surface of this cell line, providing further evidence of the role of this domain in this process (Smith *et al.,* 1998). Heterologous expression has also permitted the identification of binding epitopes of PfEMP-1 which mediate adhesion to CSA and ICAM-1, these epitopes residing within DBL-γ and DBL-β of specific *var* genes respectively (Reeder *et al.,* 1999, Buffet *et al.,* 1999, Smith *et al.,* 2000 a).

Two separate studies have shown that the DBL-α domain from two different *var* genes mediate another adhesive phenotype, rosetting (Chen *et al.,* 1998 a, Rowe *et al.,* 1997). These studies showed that parasites rosette via two different erythrocyte surface receptors.

Although there is much evidence to support the role of PfEMP-1 in adhesion, others have identified an invariant molecule, named sequestrin, which appears to mediate

adhesion of infected erythrocytes to CD36 (Ockenhouse *et al.,* 1991 b). Crandall & Sherman (1991) have also suggested that the modification of an erythrocyte protein, band 3, results in adhesion to CD36. Recent experimental evidence suggests that this adherent phenotype may be mediated via parasite modified Thrombospondin adhering to the CD36 receptor (Lucas & Sherman, 1998).

1.8 Aims

Our laboratory has a continuing interest in the *var* gene family and the proteins they encode. This gene family is estimated to represent 2-6% of the parasites genome, a substantial investment of the parasite's resources (Su *et al.,* 1995). These genes are not only important in terms of parasite biology but have been implicated in the severity of the disease. Evidence continues to accumulate suggesting that these genes mediate the cytoadherence phenotypes, which precipitate sequestration in organs such as the brain and the placenta, resulting in pathology. Immunological studies also provide evidence to suggest that the immune response against these antigens is important in the development of clinical immunity. Thus defining both the host and parasite receptors involved in this process will permit investigation in to the mechanisms of sequestration, and the immune response against the infected erythrocyte surface. Such studies will also facilitate the development of anti-adhesive therapies for use in treatment of disease.

The primary aim of this study is to try and identify the parasite ligand that mediates cytoadhesion to Chondroitin sulphate A (CSA), a host ligand implicated in the placental sequestration of infected erythrocytes. Chinese Hamster Ovary cells (CHO), which have

been developed as an adhesion model for infected erythrocyte adhesion to CSA, will be used to select for infected erythrocyte adhesion. The second aim of this study is the identification of the gene sequence of the var gene (PfEMP1) which mediates CSA adhesion. The parasite clone 3D7A is being sequenced by the Malaria Genome project. To make use of this sequence data 3D7A has been chosen as the parasite clone to be used in these assays. The third aim of this study is to investigate whether 3D7A parasites selected for adhesion to CHO cells undergo antigenic variation in a random or sequential manor.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and equipment

Standard laboratory chemicals and solvents were obtained from British Drug House (BDH) plc. and Sigma Chemical Co. Ltd., UK. and were of analytical grade. The sources of other materials are given in the text. Centrifugation was either carried out in a bench top centrifuge MSE, (Fisons) or IMV-13 microfuge (IBI). Higher speed centrifugations were performed in a Sorvall-RC-5B-refrigerated centrifuge.

2.1.2 Parasite, erythrocytes and sera

The *P. falciparum* clone 3D7A (Walliker. *et al.,* 1987) is kept in the WHO Registry of Standard Strains of Malaria Parasites in the Centre for Parasite Biology, ICAPB, University of Edinburgh. Fresh whole blood, group O Rh+, and serum used for parasite culturing was obtained from Edinburgh and South East Scotland Blood Transfusion Service. The blood was washed and centrifuged at 1500g three times in incomplete RPMI medium to remove citrate. The "buffy coat" of white cells was removed from the red cell pellet, which was resuspended in complete medium to

give a haematocrit of 50%. The washed red blood cells were kept at 4°C for up to one week (Hyde, 1993).

2.1.3 Bacterial strains

The following bacterial strains were used in the course of this study: XL1-Blue, genotype:*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*⁻ F' [*proAB*⁺ *lac*I*q*lac*Z*ΔM*15*Tn*10(tet*ʳ*)*].

2.1.4 CHO Cells

Standard K1-CHO cells and CHO cells transfected with the enocoding sequence for the ICAM-1 gene and the CD36 gene were a kind gift from Dr A. Rowe (Hasler *et al* .,1993). The CHO-745 Mutant cell line was a kind gift from Dr A. Scherf (Scherf *et al.*, 1998)

2.1.5 General stock solutions and media

All aqueous solutions were prepared in de-ionised glass distilled water. pH values of solutions were measured with a pH meter model PW 9410 (Philips).

TAE	<u>50x stock solution</u>	242g Tris base, 37.2g $Na_2EDTA.2H_20$, 57.1ml glacial acetic acid, dH_20 to 1 litre. This solution was diluted x50 to give <u>1x working solution</u> (40mM tris acetate, 2mM EDTA).

SDS <u>10% stock solution</u>	100g was dissolved in 900ml dH_20, heated to 68°C and the pH was adjusted to 7.2 by HCl, and made up to 1 litre with dH_20.
PBS <u>10x stock solution</u>	80g NaCl, 2g KCl, 11.5g $Na_2HPO_4.7\ H_20$, 2g KH_2PO_4, were dissolved in a final volume of 1 litre of dH_20. <u>working solution</u>, pH~7.3 consisted of 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7\ H_20$ and 1.4mM KH_2PO_4.
TE Buffer (1x)	10mM Tris-HCl pH 8.0,1mM EDTA pH 8.0
SOC Medium	2% Bacto-tryptone,0.5% Bacto-yeast extract,10mM NaCl, 2.5mM KCl,10mM Mg_2Cl, 10mM Mg_2SO_4,20mM glucose
Luria-Bertani Medium (LB)	1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, adjusted to pH 7.2 using NaOH
LB agar	LB medium with 1.5% Bacto-agar
LB-amp	LB supplemented with ampicillin to 50µg/ml
X-Gal	Fresh dry LB-amp. plates spread with 25µl X-Gal (40mg/ml stock solution in dimethyl-formamide).

IPTG	Stock solution (0.1M) was made by dissolving 0.238g in dH_2O, filter sterilised, and stored in 1ml aliquots at -20°C.
DNA Loading Buffer	50mM Na_2EDTA. 0.25% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose in H_2O. 20ml volumes were made up and stored as small aliquots at 4°C.
RPMI (incomplete) Medium	10.4g of RPMI 1640, 5.94g of HEPES, dissolved in 960ml of dH_2O, filtered through 0.22μM Nalgene filter and stored for up to four weeks at 4°C.

Complete RPMI Medium (parasite culture)

Prepared by addition of 42ml sodium bicarbonate, 50mg/ml gentamycin, and 40ml of heat inactivated human serum to 400ml of incomplete RPMI medium.(pH 7.2)

Complete RPMI Medium (CHO cell culture)

Prepared by addition of 42ml sodium bicarbonate, 50mg/ml gentamycin, and 40ml of heat inactivated Foetal calf serum to 400ml of incomplete RPMI medium (pH 7.2)

Binding Medium	Prepared by addition of 50mg/ml gentamycin, and 40ml of heat inactivated Foetal calf serum to 400ml of incomplete RPMI medium (pH 6.9)
Competent Cell Buffer sterilised.	60mM calcium chloride, 15% glycerol, filter Store at -70.
20 X SSPE	3.6M NaCl, 0.2 Sodium Phosphate and 0.02M EDTA pH7.7
20 X SSC	3M NaCl 0.3M Na_3 citratrate
Denaturing Solution	1.5M NaCl and 0.5M NaOH
Neutralising Solution	1.5M NaCl, 0.5M Tris-HCL pH 7.2 and 0.001M EDTA
Denhardt's Solution	2% [W/V] BSA (bovine serum albumin), 2% [W/V] Ficoll™ and 2% [W/V] PVP (ployvinalpyrollidone)

2.2 Methods

2.2.1 Culture of asexual parasites

Cultures of *P. falciparum* parasites were maintained using standard methods (Trager and Jensen, 1976; Hyde, 1993). Parasites were grown in complete RPMI 1640 medium supplemented with 10% human serum, 37.5 mM HEPES, 5% sodium bicarbonate and 25mg/ml of gentamycin sulphate. Washed human red blood cells (group O) were added to a final concentration of 5%. Red blood cells and serum were obtained from the Blood Transfusion Service. Cultures were maintained at 37^{0}C in a modified gas mixture of 96% N_2, 3% CO_2 and 1% O_2. Medium was replaced daily. Parasitaemia was determined each day by examination of blood smears on glass slides fixed with 100% methanol. Blood smears were stained with Giemsa's solution (in Sorensen's buffer, pH7.2) for 45 minutes and evaluated by light microscopy. Cultures were initiated at a parasitaemia of around 0.5% and diluted upon reaching 10%.

2.2.2 Sorbitol synchronisation of asexual parasite cultures

The parasite culture was transferred to a 15-ml tube and centrifuged at 4000 rpm for 3 mins and the medium removed by aspiration. The culture was then washed by adding 5 ml incomplete RPMI 1640 medium followed by centrifugation at 4000 rpm. The cells were then re-suspended in 5% sterile sorbitol/PBS to the same volume as the original culture and incubated at 37°C for 10 mins. The suspension

was then centrifuged at 3000 rpm and washed twice in incomplete RPMI 1640 medium. The parasites were then transferred back to culture. After 24 hours the culture was examined by blood smear, (They should contain mainly large trophozoites). Sorbitol treatment was occasionally repeated 30-36 hours after initial treatment to make the culture more synchronous.

2.2.3 Plasmagel flotation

Parasite culture was transferred to a 15ml tube, centrifuged at 3000 rpm for 3 mins and the supernatant removed. Incomplete RPMI 1640 was added to give a hematocrit of ~40%. To this an equal volume of pre-warmed Plasmagel (Bellon, France) was added. This suspension was mixed and incubated upright in a 37°C incubator for 10-30 minuets (until 2 separate layers were clearly visible). The upper layer containing Trophozoites was gently removed and transferred to a fresh tube. They were then washed in incomplete RPMI and the success of the separation assessed by blood smear.

2.2.4 Preparation of parasite DNA

DNA was extracted from healthy parasite cultures using 5ml of asexual culture at 5-10% parasitaemia. The parasites were pelleted by centrifugation at 4,000 rpm for 10 minutes. The erythrocyte pellet was resuspended in 1ml of 0.1% saponin in phosphate-buffered saline (PBS) and incubated at room temperature for 5 minutes. The erythrocyte lysate was transferred to a microfuge tube and spun for 5 minutes at 10,000 rpm. The parasite pellet was washed four times with 1ml of ice cold PBS.

Lysis of the parasite pellet was carried out overnight at 50°C with 600µl of 10mM Tris-HCL pH 7.6, 50mM EDTA pH 8.0, 0.1% SDS, 1mg/ml proteinase K. The lysate was extracted twice with phenol/chloroform, then once with chloroform. DNA was precipitated for 15 minutes at room temperature by the addition of an equal volume of isopropanol and 10% volume of 5M sodium acetate. DNA was pelleted by centrifugation at 15,000 rpm for 5 minutes, washed with 70% ethanol and resuspended in 100µl of PCR grade.

2.2.5 Preparation of parasite RNA

A flask of ring stage parasite culture was pelleted by centrifugation at 4000g for 5 minutes. The pellet was then re-suspended in 1ml of 0.1% saponin in phosphate buffered saline (PBS) and incubated at 37°C for 10 mins. The parasite lysate was then transferred to a microfuge tube and washed X3 in 10ml ice cold PBS. The parasite pellet was then re-suspended in 1ml RNA isolator ™ (Genosys) the RNA was subsequently isolated according to the RNA isolator protocol. Essentially 0.2ml of chloroform was added to the parasite /RNAisolator mix. the suspension was gently mixed for 15 seconds and allowed to stand for 10 mins. The eppendorf was then centrifuged at 12,000g for 15 mins at 4°C. the aqueous phase (which contains RNA) was removed. To this 0.5ml isopropanol was added. This was again allowed to stand for 10mins before being centrifuged at 12,000g, 4°C for 10 mins.

The RNA pellet was then washed in 75% ethanol and re-suspended in 0.2ml H_20. To remove contaminating DNA, the RNA was subjected to DNAse treatment . 5µl (50units) of DNAse1, RNAse-free (Boehinger Mannheim) was added to the RNA which was subsequently incubated for 30 mins at 37°C. The enzyme was then

extracted by adding 30µl phenol chloroform. Followed by centrifugation at 12,000g for 2 mins. The top aqueous layer was removed and placed in a fresh tube. To this 30µl chloroform was added and again the eppendorff was centrifuged at 12,000g for 2 mins. The top aqueous layer was removed and transferred to a fresh tube. 1ml 100% ethanol was added and the tube was then centrifuged at 12,000g , 4°C for 10 mins. The ethanol was removed by aspiration and the RNA pellet washed by adding 1ml 75% ethanol, centrifugation at 12,000g for 5 mins and aspiration of the supernatant. The RNA pellet was then re-suspended in 30µl H_20.

2.2.5 Preparation of plasmid DNA

Plasmid DNA was prepared either by the SDS-alkaline extraction method as described by Sambrook *et al.* (1989) or using the QIAprep Spin Miniprep kit (Qiagen Inc.) according to the manufacturers instructions. Briefly, a single colony of transformed *E. coli* was grown overnight at 37°C in 5 ml of LB broth. Cells were pelleted by spinning for 5 minutes at 2,000 rpm, and the medium discarded. The pellet was then lysed and plasmid DNA isolated following the manufacturers instructions.

2.2.6 DNA quantification

The concentration of DNA was estimated by measuring its absorbance at 260nm in a DNA quant (Eppendorf). It was assumed that an OD_{260} of 1.0 is equivalent to a concentration of 50µg/ml for double stranded DNA and 35µg/ml for oligonucleotides.

2.2.7 Restriction enzyme digestion

Restriction enzyme digests were performed using the appropriate buffers supplied by the manufacturers (Boehringer Mannheim and Promega). Reactions were carried out at 37°C for 3-6 hours, using 4-12 units of enzyme per µg of DNA. Reactions were stopped by the addition of 10% volume of 50mM Na_2EDTA, 40% w/v sucrose, containing 0.25% bromophenol blue and 0.25% xylene cyanol.

2.2.8 cDNA synthesis

The cDNA was reverse transcribed from total RNA using the Expand™ Reverse Transcriptase kit (Roche) following manufacture's instructions. 2.5µl degenerate var3 primer was added to 3µl total RNA which was then incubated at 65°C for 10 minutes then placed on ice. To this 4 µl Expand reverse transcriptase buffer, 1mM dNTPs, 20 units of Rnase inhibitor and 50 Units of Expand™ Reverse Transcriptase was added. This was made up to 20µl with water. The mixture was transferred to a heat block and incubated at 42°C for 60 mins. The sample was then transferred to ice and subsequently used for PCR or stored at -70°C.

2.2.9 Polymerase chain reaction (PCR)

PCR is used for the enzymatic synthesis of DNA sequences using a thermostable DNA polymerase such as *Taq* (Saiki *et al.*, 1988). DNA is first denatured, then annealed with specific primers corresponding to each end of the target sequence, and finally the annealed primer sequences are extended towards each other until

they meet. This cycle is repeated 20-40 times and potentially amplifies the target sequence by $x10^7$-10^9 fold.

Standard PCR reactions of 20-50µl volume contained 1/10 volume of 10 x buffer (Roche), 1/10 volume of 10µM of each of forward and reverse primers, 1/10 volume of 750µM dNTP, 1/100 volume of *Taq* polymerase (Roche) around 1/10 volume of template (dependent upon DNA concentration), and PCR grade water to final volume. Standard PCR conditions used unless otherwise indicated in the text were, 1 initial cycle of 93^{0}C for 1 minute, followed by 30 cycles of 93°C for 30 seconds, 50°C for 30 seconds and 65°C for 5 minutes, then a final cycle of 65°C for 10 minutes. Negative controls containing no DNA template were included in each set of reactions. The products of PCR reactions were analysed using agarose gels electrophoresis.

2.2.10 Automated DNA sequencing

Automated sequencing of plasmid DNA was carried out using dye terminators with the PRISM™ cycle sequencing kit (Applied Biosystems Inc.) and the 377 automated sequencer (ABI). Reactions were performed as per the manufacturers instructions modified to a final volume of 10µl (ABI PRISM Dye Terminator protocol, P/N 402078). For each reaction, the reagents were aliquoted into a 0.5ml PCR tube as follows: 4µl Terminator Ready Reaction Premix, 2 µl dsDNA Template (0.2µg/ml), 2µl sequencing primer (0.8 pmol/ml), and 2µl PCR grade H_2O. Cycle sequencing was performed on the HYBAID DNA thermal cycler (Omnigene) with the following conditions: 25 cycles (96°C for 30 sec, 50°C for 20 sec, 60°C for 4 min, respectively), then kept on 4°C for 10 mins. PCR products

were precipitated, washed and resuspended in 2.5µl deionized formamide and 0.5µl of 50µg/ml Blue dextran in 25mM EDTA, pH 8.0. Each sample was then heated at 90°C for 5min and 1.8µl of each sample was loaded onto a 6% denaturing gel and run at 50W for 7h. Chromatograms were viewed and data edited using the Seqed™ program (ABI). Standard sequencing primers were used for the majority of reactions, with a DBL specific sequencing primer (F3) being used where necessary to complete "gaps" in the data. Obtained sequences were confirmed as DBL sequence by BLAST analysis.

2.2.11 Agarose gel electrophoresis and photography

Agarose gels were used to check the quality of genomic DNA and plasmid preparations and to analyse PCR products and restriction digestions. 1-2% agarose (IBI) was dissolved in 1x TAE buffer by boiling. After cooling to around 45°C, ethidium bromide was added to a concentration of 0.5µg/ml and the mix poured onto an electrophoresis gel plate and left to set at room temperature. The appropriate amount of DNA was mixed with 0.1 volume of gel loading buffer (40% w/v sucrose, 50mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) and loaded into the submerged gel. Electrophoresis was carried out in 1xTAE at 100 volts for an appropriate time dependent upon the size of the fragments under analysis. Fluorescence from DNA-bound ethidium bromide was visualised by short wave-length UV light and photographed.

To aid in estimation of DNA fragment size, 2µg of DNA markers were loaded adjacent to DNA samples on agarose gels. The markers used were Boehringer

Mannheim DNA Molecular Weight Marker VII. Molecular Weight Marker VII contains DNA fragments of size 8576, 7427, 6106, 4899, 3639, 2799, 1953, 1882, 1515, 1482, 1164, 992, 710, 492 and 359 bp.

2.2.12 Purification of DNA fragments from agarose gels

DNA fragments from PCR reactions or restriction enzyme digestion were purified using one of the following methods:

Phenol/chloroform extraction: Electrophoresis of the DNA fragments was carried out using a gel made from low melting temperature agarose (IBI) and 1x TAE buffer. DNA fragments were visualised by fluorescence of DNA-bound ethidium bromide under long wave UV light (366nm) and excised. The gel slice was placed in a 1.5ml microfuge tube and broken into small pieces. An equal volume of phenol/chloroform was added and the mix was vortexed for a few seconds before incubation at –70°C for 10 minutes. The frozen mixture was spun at 13,000 rpm for 5 minutes and the aqueous upper phase removed to a clean microfuge tube. Extraction was carried out first with an equal volume of phenol/chloroform/3-methyl-1-butanol (50:48:2) and secondly with an equal volume of chloroform. DNA was precipitated by addition of 0.1 volume of 3M sodium acetate and 2 volumes of 100% ethanol, washed with 70% ethanol and finally re-dissolved in PCR grade water.

Magic PCR Preps™ DNA purification system: DNA contained in low melting temperature agarose gel slices was also purified using the Promega Magic PCR

Preps™ DNA purification system according to the manufacturers instructions. The gel slice was incubated in a 1.5ml microfuge tube at 70°C until the agarose completely melted. 1ml of resin was added, mixed immediately, and the mix was passed through a mini column by syringe. The minicolumn was washed with 2ml of 80% isopropanol and centrifuged for 20 seconds at 13,000 rpm. The minicolumn was placed in a clean microfuge tube and 50µl of PCR grade water was applied to the column and incubated for 3 minutes. The microfuge tube was spun at 13,000 rpm for 1 minute to elute the DNA.

2.2.13 Preparation of competent bacterial cells

The method used was modified from Hanahan (1983). Cells of the appropriate *E. coli* strain were plated on LB agar overnight at 37°C. A single colony was picked and grown overnight in 5ml LB broth at 37°C with shaking. The following morning, the culture was diluted 50-fold in LB broth and incubation continued for approximately 2-4 hours until the cell density was 4-7x10^7 viable cells/ml (OD_{600} of 0.4-0.6). At the same time, 150ml of 60mM calcium chloride in 15% glycerol was prepared and sterilised by filtering through a 0.22µm filter and kept on ice. The culture was then chilled on ice for 10 min and pelleted at 4,500 rpm for 5 min at 4°C. The pellet was resuspended in 50ml of the ice-cold calcium chloride/glycerol solution, and incubated on ice for 5min then re-pelleted as above. The pellet was resuspended for a second time in 50ml of ice-cold calcium chloride/glycerol and incubated on ice for 30min. The cells were pelleted once more as before and resuspended in 5ml of the ice-cold calcium chloride/glycerol solution and 100-

200µl volumes aliquoted into sterile pre-chilled microfuge tubes. The competent cells could be stored at -70 C for up to 2 months, or used directly.

2.2.14 Transformation of *E. coli*

Competent cells (50-100µl) were mixed with either 5µl of a ligation reaction or 20ng of uncut plasmid DNA. The cells were then incubated on ice for 30 minutes, heat-shocked at 42°C for 60 seconds and incubated in ice for 2 minutes. To each transformation, 400µl of pre-warmed SOC medium was added and the mixture was incubated at 37°C with gentle shaking for 1 hour. The cells were pelleted by brief centrifugation in a microfuge and all but 100ml of the medium removed. The cells were resuspended in the remaining medium and 25-100µl plated out on appropriate selective LB agarose plates. Plates were incubated overnight at 37°C.

2.2.15 Identification of clones of interest

Transformed cells were plated out on LB plates containing appropriate antibiotic to select for the presence of plasmid. Where appropriate, α-complementation was used to detect the presence of insert DNA. The method used was that presented in Sambrook *et al.*, 1989. Briefly, 40µl of 20mg/ml X-Gal and 40µl of 20mg/ml IPTG were added to LB plates before plating of the transformants. Following overnight incubation at 37°C, the plates were incubated at 4°C for 2-4 hours to allow colour development. Using this method colonies containing inserts (white) can be distinguished from colonies without insert (blue).

Recombinant clones were further analysed for the presence of inserts of desired size by one of the four following methods :

Direct size comparison of plasmid minipreps and control vector on agarose gels.

Restriction enzyme digestion of plasmid minipreps.

PCR amplification using plasmid minipreps as DNA template using either specific *var* primers or vector derived primers to amplify inserts.

Direct PCR performed on lysates of bacterial colonies. A sterile pipette tip was touched to a bacterial colony surface and then shaken in 50µl of PCR grade water in a microfuge tube. The microfuge tube was heated to 100°C for 5 minutes and then spun for 5 minutes at 13,000 rpm. 5µl of supernatant was then used as template in a standard PCR assay.

2.2.16 Sequence Alignments

Deduced amino acid alignments of DBLα sequences were made using the GCG pileup and MacVector clustal functions Sequences were aligned with gap weight of 3.0 and gap length weight of 0.1 and edited by hand.

2.2.17 Dot Blot hybridisation

Quantified plasmid DNA was denatured on a heat block at 95°C then chilled on ice. This was added to 1 volume 20 X SSC. 10µl DNA was then spotted on to a nylon membrane pre-wetted with 10X SSC using a vacuum supplied blotter. The membrane was transferred to a tray of denaturing solution for 5 minutes, before being transferred to neutralising solution for 1 minute. The DNA was then fixed to the membrane by exposure to UV for 2 mins (each side)

2.2.18 Southern Hybridisation

Pre-hybridisation solution was made up following manufacture's instructions to a final concentration of 5X SSPE, 5X Denhardt's solution 0.5% SDS and 5mg salmon sperm DNA. The nylon membrane (Amersham) was incubated in pre-hybridisation solution for 60 minutes at 65°C. The probe, labelled by random priming according to manufacture's instructions (Roche) was denatured at 100°C for 5 mins and incubated with the membrane at 65°C over night (minimum 12 hours). Following hybridisation, the membranes were washed twice with 2X SSPE, 0.1% SDS for 15 minutes at 65°C, 1X SSPE, 0.1% SDS for 15 mins at 65°C and 0.1X SSPE, 0.1% SDS for 15 minutes.

2.2.19 Chinese Hamster Ovary Cell culture

Thawing CHO cells

A vial of CHO cells was removed from liquid nitrogen (or –70) and transferred to a 37°C water bath until thawed. The cells were transferred to a 15ml tube and re-suspended in 10ml complete medium. The cells were centrifuged at 2000 rpm and the supernatant removed. The cell were again re-suspended in 5 ml complete medium and transferred to a small flask and incubated in a C02 incubator (5% C02) at 37°C.

Growing cells

Cell become confluent after 3-4 Days. Medium was changed by aspirating off the old medium and replacing with 5ml fresh complete medium when cells were confluent or every 72 hours. Progress of the culture could then be viewed under an inverted microscope.

Splitting cultures for continuous growth

When cells become confluent they were split by removing old medium by aspiration and washing the cells twice in 10 ml incomplete medium, followed by addition of 1.5 ml of pre-warmed 2mM EDTA/PBS (medium size flask, 4ml, large flask, 7ml). This was then incubated for 5-10 mins at 37°C. The cells were dislodged by gentle tapping of the side of the flask. The cells plus medium were removed and placed in a 15ml tube and centrifuged at 2000 rpm. The supernatant was then removed and the cells re-suspended in 10ml complete medium. The cells were then seeded into fresh flasks. When transferring into a medium sized flask 25ml medium was used and 40ml for large flasks. Cells were counted using a haemocytometer. 5-10µl of re-suspended cells were place on the haemocytometer

and the number of cells in 2 large squares was counted (average = Z) the number of cells equalling Z x ^{104}/ml.

Freezing cells

Cells to be frozen were washed in complete medium and counted as above. 1 X ^{106}/ml cells were re-suspended in complete medium + 10% DMSO, mixed well and transferred to –70 overnight and then to liquid nitrogen.

2.2.20 CHO cell selection assay

The selection assays were conducted essentially as described by Scherf *et al.*, 1998. A large flask of CHO cells was cultured until the flask was semi-confluent. The cells were then subjected to 3-4000 rads of gamma radiation (this reduces the contamination of parasite cultures with CHO cells after selection). The cells were then washed twice in binding medium (pH6.9). The parasite culture was treated with Plasmagel to concentrate the mature parasite stages (see above). The trophozoite pellet was washed twice in binding medium and resuspended in 15 ml binding medium. The cells were then transferred to the flask of semi-confluent CHO cells and allowed to bind. The flasks were incubated for 60 minutes in a C02 incubator at 37°C, with agitation every 15 mins. The unbound parasites were gently washed off by 7-9 washes with 10 ml binding medium. Success of selection could sometimes be assessed using an inverted microscope. 0.5ml of fresh blood and 15ml complete parasite culture medium was then added and the flask gassed and incubated as a parasite culture. 24 hours later the culture was transferred into a fresh culture flask and the parasitaemia assessed by blood smear .

2.2.21 CHO cell adhesion assay

CHO cells were cultured in a 30ml culture dish until semi confluent. The culture was then washed twice in binding medium. A culture of predominantly trophozoite stage parasites at parasitaemia ~5-10% was transferred to a 15ml tube and washed twice in binding medium. The parasite culture was then re-suspended to a hematocrit of 5%. 3ml of culture was added to the culture dish which was incubated in a $C0_2$ incubator for 60 minuets at 37°C. with gentle agitation every 15 mins. The culture was then washed 5 times with binding medium and fixed for 30 minutes in 1% gluteraldyhyde. The culture dish was then stained with 2% filtered Giemsa for 30 minutes. The bound cells were then counted under oil immersion.

2.2.22 Trypsin digestion

Trypsin treatment was conducted essentially as described in Gardener *et al.*(1996). A large parasite culture at 5-10% parasitaemia was prepared for binding, transferred to a 15ml tube and washed twice in incomplete RMPI 1640 medium. Trypsin was added to a concentration of 100µg/ml (Sigma) and the parasites incubated for 10 mins. at 37°C. The parasites were then used in the binding assay.

2.2.23 Enzymatic digestion of CHO cells.

$1X10^5$ CHO cells were transferred into a 15 ml tube and washed twice in incomplete RPMI medium. Enzyme (chondroitinase ABC, hyaluronidase, heparinase, or neuraminidase,) was added to a concentration of lU/ml. Cells were incubated at 37°C for 1 hour. The cells were then washed, resuspended in binding medium and used for the binding assay.

2.2.4 Inhibition of adhesion with glycosaminoglycans

CHO cells were prepared for the adhesion assay as above. Parasites prepared for binding were transferred into a 15 ml tube in binding medium to a 4% hematocrit and gylcosaminoglycans (chondroitin sulphate A, chondroitin sulphate C, heparane sulphate, hyaluronic acid or neuraminic acid) were added to a concentration of 25mg/ml. Cells were then incubated for 15 mins at 37°C before being added to the petri dish for the binding assay.

Chapter 3

Development of a model system for studying cytoadhesion of *P.falciparum* infected red blood cells: selecting 3D7A lines which bind Chinese Hamster Ovary Cells

3.1 Introduction

Post mortem microscopy first revealed that concentration of *P.falciparum* infected erythrocytes in the small blood vessels of vital organs appeared to be due to adhesion of infected erythrocytes to capillary and post-capillary endothelial tissue. Since then a great deal of effort has gone into understanding this phenomenon.

The modern analysis of parasite cytoadhesion and sequestration has centred on the development of *in vitro* assays of cytoadherence. Some of these involve measurement of the ability of infected erythrocytes to adhere to purified protein or candidate carbohydrate adhesion receptors. Such receptors are simply electro-statically bound to polystyrene micro-culture plates. Other adhesion assays have been developed which attempt more realistically to reproduce adhesion receptor presentation and *in vivo* cellular interactions. Such assays may simply involve counting infected erythrocytes which adhere to *in vitro* cultured cell lines such as HUVEC or C32 melanoma cells. More "controlled" types of cellular adhesion assay involve the use of cell lines transfected with particular candidate receptor sequences carried on plasmid expression vectors. These constructs produce large amounts of the particular cell surface antigen expressed at the surface of the transfected cell. In this way exogenous receptors can be

inserted into a 'background' cell surface normally lacking certain known candidate *P. falciparum* adhesion receptors. The most frequently used example of such a system are Chinese hamster ovary (CHO) cells transfected with intracellular adhesion molecule-1 (ICAM- 1) and the endothelial marker and leukocyte differentiation antigen CD36 (Hasler *et al.,* 1993).

To date, the protein or glycoprotein cell surface antigens CD36, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), E-Selectin, P-Selectin, PECAM-1, thrombospondin and complement receptor 1 have all been identified as potentially important infected erythrocyte adherence receptors. The glycosaminoglycans, chondroitin sulphate-A, hyaluronic acid and heparan sulphate have also been identified as potential receptors (Rogerson *et al.,* 1995, Beeson *et al.,* 2000, Chen *et al.,* 1998b). Under some conditions, probably under the influence of host factors such as cytokine release, cytoadhesion and tissue sequestration events result in clinical consequences. Some of these may be relatively mild such as malaise, cough, diarrhoea and myalgia. Others may be more severe and include cerebral malaria, renal and hepatic impairment and metabolic acidosis. Pregnant women who contract malaria may suffer severe complications such as intrauterine growth retardation, premature delivery and perinatal mortality (Rogerson & Beeson, 1999 b). Largely due to the identification of CSA as a placental adhesion receptor, pregnancy-associated malaria has become a topic of considerable interest in malaria research (Fried & Duffy, 1996, 1998)

A major objective of this project was to study the interaction between a *P. falciparum* parasite population known to be undergoing continuous antigenic variation and CSA.

Since an *in vitro* model of cytoadhesion would seem to be most analogous to the natural situation it was decided to select sub-populations of the *P. falciparum* clone 3D7A capable of binding to the surface of CHO cells. CHO cells are known to express high levels of chondroitin sulphate A (Rogerson *et al.,* 1995). They therefore appeared to offer a particularly good *in vitro* model system for the analysis of PfEMP-1 mediated binding to CSA.

3D7A (Walliker *et al.,* 1987), a clone derived from the NF54 (*Plasmodium falciparum*) strain isolated from a Dutch malaria patient thought to be suffering from imported 'airport' malaria, is one of many cloned parasite lines kept in the WHO Registry of Standard Strains of Malaria kept in Edinburgh University. It is also the parasite clone being sequenced by the Malaria Genome Project. Investigating the molecular basis of cytoadhesion of this particular clone would therefore permit exploitation of the sequence data being generated by the genome project in identifying the parasite derived protein or proteins involved.

3.2 Results

3.2.1 Selection of adherent lines from the parasite clone 3D7A

To start the selection process, parasites previously synchronised by 5% sorbitol treatment were grown to 10% parasitaemia, with most parasites at the trophozoite developmental stage. These mature stages were increased in concentration using the Plasmagel flotation technique . The degree of enrichment for trophozoites was assessed

by examining a thin blood smear, which normally indicated that 80-90% of the concentrated erythrocytes were infected with mature trophozoites. These parasites were further concentrated by centrifugation and the final trophozoite pellet was washed in binding medium and stored at 37°C prior to selection.

CHO cells to be used for selection were cultured until semi-confluent. The culture flask was then treated with 3000-4000 rads of gamma-radiation to kill the cells prior to selection. This was done because the use of live CHO cells in a selection assay can result in the transfer of some of these cells into the parasite culture after selection. These Two cell types compete for nutrients in the same medium, usually resulting in poor parasite growth. Irradiation of CHO cells prior to selection limits the subsequent contamination of selected parasite cultures with live CHO cells (Rogerson *et al.,* 1995).

The irradiated CHO cells were then washed with binding medium to remove unbound CHO cells. The Plasmagel-purified trophozoites were then transferred into the CHO cell flask and resuspended in binding medium (see Methods). After allowing the infected red cells to bind to the CHO cells for 1 hour the culture supernatant containing unbound erythrocytes was aspirated off and the flask washed several times with binding medium to remove any unbound erythrocytes from the flask. The CHO cell-adherent erythrocytes were then viewed unstained under an inverted microscope. 500µl fresh erythrocytes (50%v/v) were then added to the culture flask. Merozoites released from cytoadherent parasites infect a proportion of these erythrocytes during the over night incubation. The parasite-containing supernatant was then cultured to a parasitaemia of 10% after subdivisions into two large culture flasks. The process was repeated 6-8 times before the

level of binding was assessed.

3.2.2 Binding assay for parasites selected for adhesion to CHO cells

CHO cells were added to 30mm culture dishes and grown until semi-confluent. The cells were then washed in binding medium and stored at 37°C ready for binding. To measure the relative level of binding of selected versus unselected 3D7A lines, synchronised parasite cultures (at the trophozoite stage) were washed in binding medium and resuspended to a 4% hematocrit. 3 mls of parasite suspension were allowed to bind for 1- hour, with resuspension by hand every 15 mins during this period. After completing the binding assay the dishes were washed 5 times with binding medium to remove unbound erythrocytes and then fixed by the addition of 1% gluteraldyhyde for 30 mins. They were then stained with 1% Giemsa for 30 minutes. The dishes could then be observed under X 100 objective to count the number of bound infected erythrocytes per 1000 CHO cells (Fig. 3.1).

Figure 3.1 Selected 3D7A infected erythrocytes bound to Chinese Hamster Ovary cells (magnification X400)

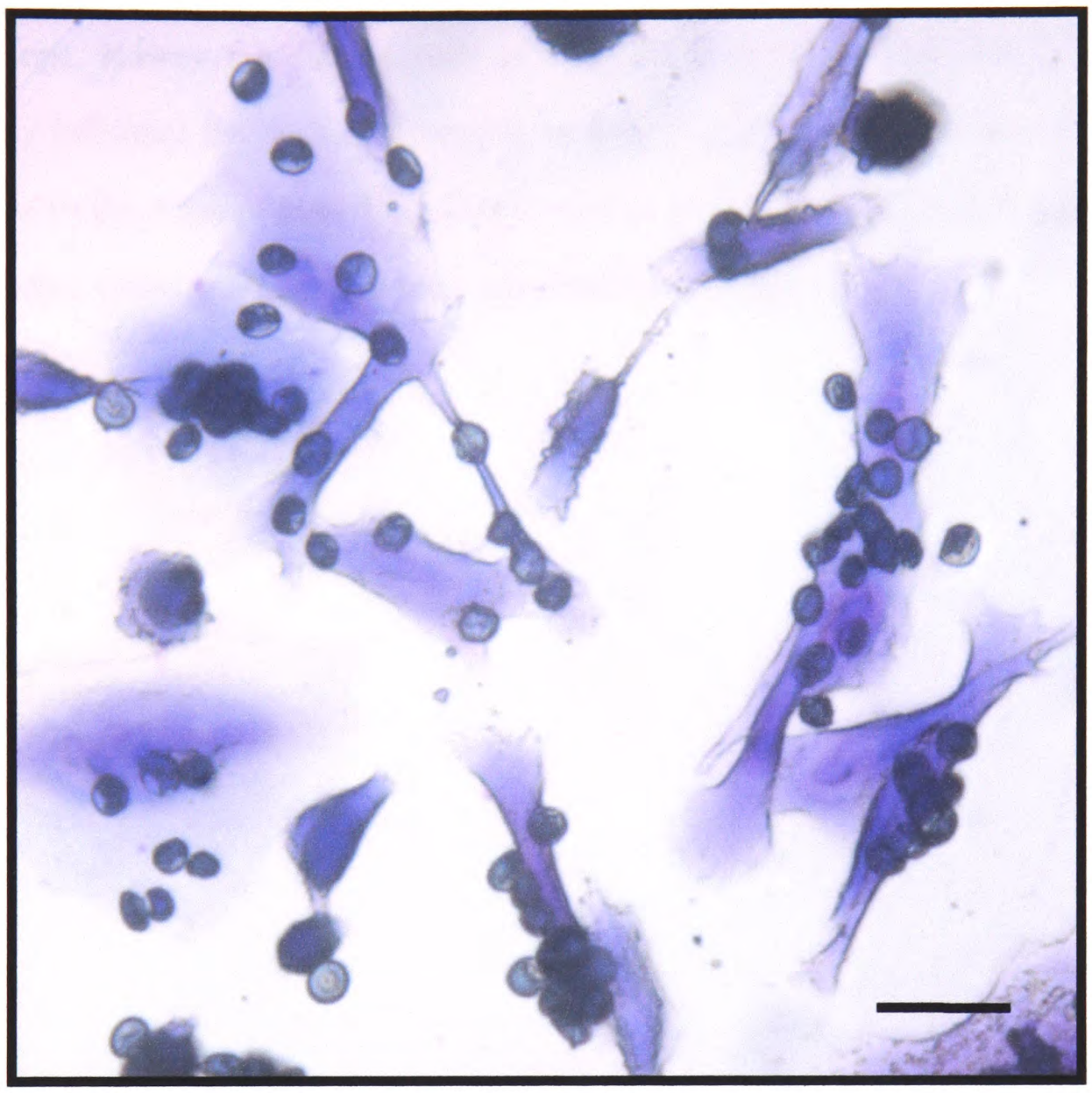

Figure 3.1 Geimsa stained binding assay. Selected 3D7A parasites (small dark violet stained spheres) adhering to CHO cells (large violet stained cells). Size bar 20μm.

The binding experiments were conducted in duplicate on 3 separate occasions. All binding experiments were carried out blind (i.e. selected and unselected parasites were coded). The degree of binding between duplicate experiments was consistent. However,

there was variation between experiments conducted on different days with different batches of CHO cells and parasites, which led to a large standard deviation (Table 3.1). The parasite cultures were normally used for binding at 5-10 % parasitaemia and 4% hematocrit. However variables such as the confluence and condition of CHO cells probably influence the degree of parasite binding. In addition the adhesion phenotype is sensitive to the stage of infecting parasite, and so variation in the stage of parasite used in a binding assay may also influence variability in binding.

Figure 3.2 Selected 3D7A infected erythrocytes adhering to CHO cells (magnification X1000)

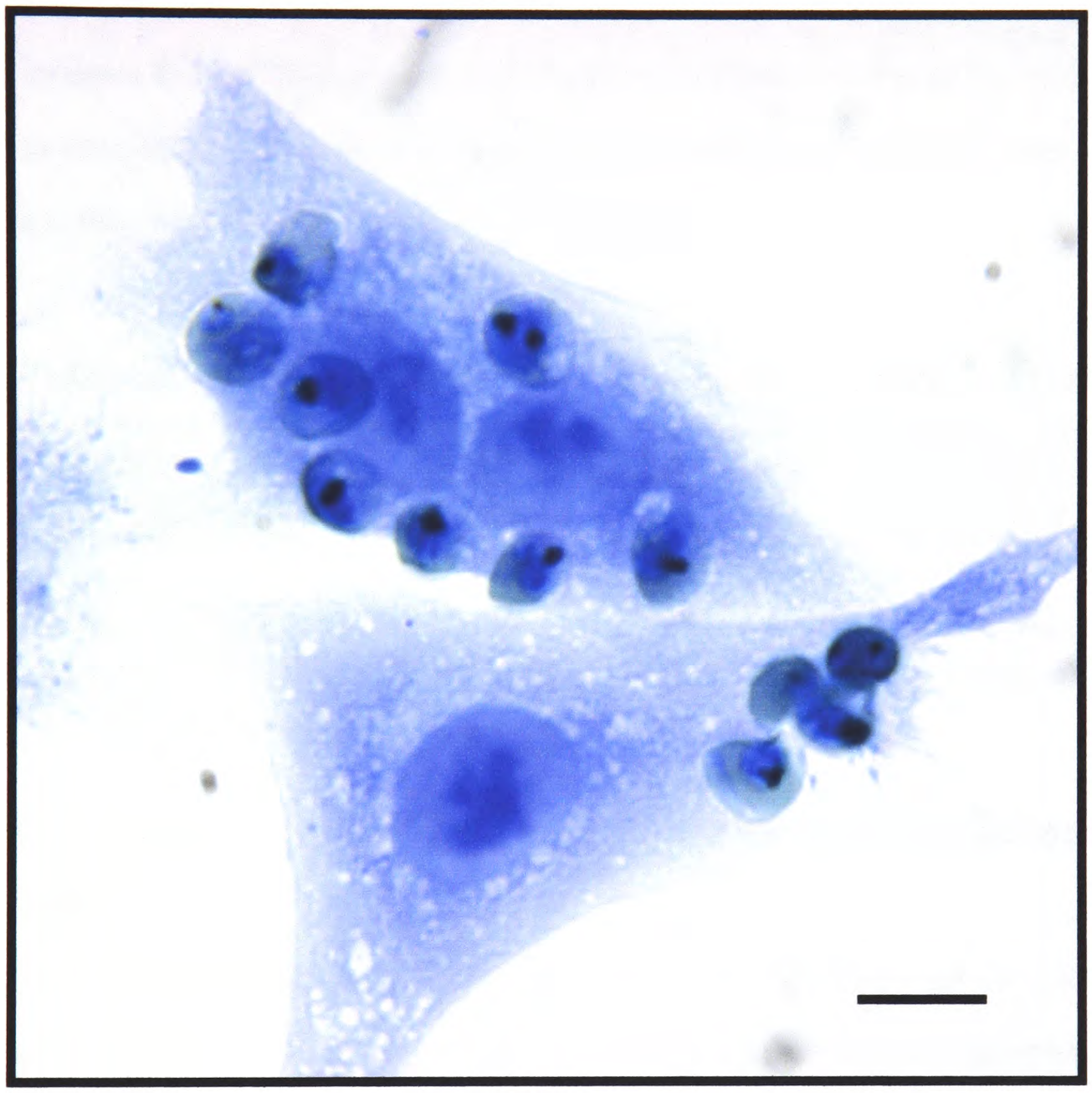

Figure 3.2 Giemsa stained binding assay (magnification X1000). Selected 3D7A parasites (small dark blue stained spheres) adhering to the surface of 2 CHO cells (large blue stained cells). Size bar 10μm.

The numbers of unselected 3D7A parasites which were capable of binding to CHO cells was very low, being approximately 1-2 parasites per 1000 CHO cells. After 8 rounds of selection binding to CHO cells had increased by over 2 orders of magnitude (Fig. 3.2 & Table 3.1), between 2-20 parasites being found bound to the surface of each CHO cell (Fig. 3.1). This increase was found to be statistically significant using a one tailed students T-Test (P= 0.00078). This highly significant increase in the selected versus the unselected line showed that selection had clearly enriched the proportion of parasites capable of binding to the surface of CHO cells.

Table 3.1 binding of selected 3D7A parasites per 1000 CHO cells

PARASITE TYPE	3D7A UNSELECTED	3D7A SELECTED
Number of parasites	3.5 ±4.1	1014 ±144.2

Table 3.1 Binding of selected and unselected parasites to CHO cells, parasites counted per 1000 CHO cells (selected parasites subjected to 8 rounds of selection). N = 3, ± = standard deviation.(T Test P= 0.00078)

3.2.3 Trypsin sensitivity of infected erythrocyte cytoadhesion to CHO cells

Having established that a CHO cell binding line of 3D7A can be selected, the next question to be answered was whether the observed increase in binding was mediated by a parasite derived molecule expressed at the erythrocyte surface, such as PfEMP-1, Prior experimental evidence suggests that cytoadhesion mediated by PfEMP-1 is disrupted by treatment of the mature infected erythrocyte with the proteolytic enzyme trypsin. Different PfEMP-1 variants have shown variable levels of sensitivity to trypsin digestion, although treatment of parasites with trypsin concentrations in excess of

100μg/ml will abolish binding in most cases (Gardener *et al.*, 1996; Baruch *et al.*, 1996).

The CHO cell binding 3D7A line was therefore subjected to trypsin treatment prior to binding. Experiments assaying the trypsin sensitivity of the adhesive phenotype were conducted in duplicate and repeated on 3 separate occasions. Parasites were incubated in the presence of 100μg/ml for 10 mins. The parasites were subsequently washed in binding medium before being used in a binding assay. The trypsin treatment reduced binding of mature infected erythrocytes to CHO cells by >95% (P= 0.00528, Table 3.2). The cytoadhesion that had occurred during the eight rounds of selection therefore appeared to be mediated by a trypsin sensitive protein present at the surface of *P. falciparum* infected erythrocytes.

Table 3.2 Trypsin treatment of selected 3D7A parasites adhering to CHO cells

PARASITE	*NUMBER OF BOUND PARASITES PER 1000 CHO CELLS*
3D7A selected	*573 ±291*
3D7A unselected	*12.5± 6.1*

Table 3.2 Results of binding of 3D7A parasites treated with trypsin versus no untreated controls to CHO cells. Experiments repeated 3 times in duplicate, N=3, ± refers to the standard deviation. (T Test, P = 0.00528)

3.2.4 Experiments to identify the CHO cell adhesion receptor by inhibition of infected erythrocyte binding with soluble carbohydrates

The apparent lack of expression of a number of major cell surface antigens by CHO cells means that they are often used as host cells in mammalian transfection experiments. For example they have been used to express the human endothelial receptors ICAM-1, CD36, VCAM-1, E- Selectin, P- Selectin, PECAM-1 and CR1. Antibody labelling experiments suggest that CHO cells do not constitutively express any of these endothelial receptors (Hasler *et al*, 1993; Johnston *et al*, 2000; Crutchfield *et al.*, 2000; Raingner *et al.*,1997 Makrides *et al.*, 1992 Pouvelle *et al.*, 2000). However CHO cells are known to express a number of carbohydrates at their surface. These include glycosaminoglycans (GAGs), which are un-branched polysaccharide chains composed of repeating disaccharide units, one of these units being an amino sugar. The only *P. falciparum* cytoadhesion receptor identified to date expressed at the CHO cell surface is the GAG chondroitin sulphate A (Rogerson *et al.*, 1995).

It has been previously demonstrated that pre-incubation of parasites in 10μg/ml CSA is sufficient to inhibit CSA mediated cytoadhesion of infected erythrocytes to CHO cells (Rogerson *et al.*, 1995). Thus, to test whether CSA was metiating this adhesive phenotype , the parasite suspension was pre-incubated in soluble CSA (extracted from porcine rib cartilage). Soluble CSA was added at a concentration of 10μg/ml. This failed to inhibit adhesion of selected 3D7A infected erythrocytes to the CHO cell surface (data not shown). The inhibition assay was repeated, pre-incubating CHO cells in the presence of

25µg/ml soluble CSA. This increased concentration of CSA also failed to inhibit adhesion (Table 3.3 ,P= 0.0037).

Chondroitin sulphate C (CSC), (extracted from shark cartilage) is very similar in structure to CSA being sulphated at carbon six in the β-glactosamine ring and not carbon four which is sulphated in Chondroitin sulphate A. This molecule represents a possible cytoadhesion receptor as experiments have shown that CSC inhibits binding of infected erythrocytes to CHO cells at very high concentrations. However is has been suggested that this could be due to the small amount of contaminating CSA in most commercial CSC preparations (Rogerson *et al.,* 1995). To test the binding inhibition capacity of CSC, parasites were pre-incubated in 25µg/ml CSC prior to being used in a binding assay. CSC also failed to inhibit adhesion of selected 3D7A infected erythrocytes to CHO cells (Table 3.3, P = 0.004917).

Heparan sulphate, although not associated with cytoadherence, has been identified as a rosetting receptor mediating the binding of uninfected erythrocytes to infected erythrocytes. It is possible that if this GAG can mediate the adhesion of infected erythrocytes to uninfected erythrocytes then it could also mediate the interaction between the infected erythrocyte and the CHO cell surface. To test this hypothesis the 3D7A line selected for adhesion to CHO cells was pre-incubated in heparan sulphate at a concentration of 25 µg/ml before being allowed to adhere to the CHO cell surface. However the addition of heparan sulphate failed to inhibit binding of selected 3D7A infected erythrocytes to the CHO cell surface (Table 3.3,P = 0.02993).

Hyaluronic acid has also recently been described as a cytoadherence receptor for infected erythrocyte cytoadhesion and is known to be expressed at the surface of CHO cells (Esko *et al.,* 1991). A selected line of 3D7A was pre-incubated in the presence of 25 μg/ml hyaluronic acid, but again no inhibition of selected 3D7A infected erythrocyte cytoadhesion was observed (Table 3.3, P = 0.0072).

N-acetylneuraminic acid (Sialic acid) is not a GAG and has not been identified as a cytoadherence receptor, but in *P. falciparum* malaria, merozoites bind to neuraminidase sensitive sialic acid in order to invade the erythrocyte (Adams *et al.,* 1992). Furthermore another protozoan parasite, *Trypanosoma cruzi,* attaches to and invades mammalian cells via sialyl residues at the cells surface (Ming *et al.,* 1993). This use of sialic acids for adhesion is not restricted to protozoa. Enteropathogenic *E. coli* and Adenovirus type 37 also use sialic acid as an adhesion receptor (Vanmaele *et al.,* 1994; Arnberg *et al.,* 2000).

Sialic acid is another carbohydrate expressed at the surface of CHO cells and could possibly represent a receptor mediating *P. falciparum* cytoadhesion. To test this hypothesis a selected isolate of 3D7A was pre-incubated in the presence of N-acetylneuraminic acid prior to binding. This carbohydrate also failed to inhibit adhesion of selected 3D7A infected erythrocytes to the CHO cell surface (Table 3.3, $P = 6.5 \times 10^{-5}$).

Throughout all of these experiments pre-incubation with trypsin was used as a control for inhibition. This treatment consistently reduced adhesion of selected 3D7A infected erythrocytes by approximately 95% (Table 3.3, P = 0.00194).

Table 3.3 Inhibition of binding of selected 3D7A to CHO cells in the presence of soluble carbohydrates

carbohydrates	PARASITES PER 1000 CHO CELLS)	T TEST (P=)
Heparan sulphate (25 μg/ml)	617.8 ± 541.3	0.02992
Hyaluronic acid (25 μg/ml)	630.3 ± 408.9	0.0072
Chondroitin Sulphate A (25 μg/ml)	509.6 ± 279.1	0.00376
Chondroitin Sulphate C (25 μg/ml)	652 ± 384	0.004917
N-acetylneuraminic acid (Sialic acid) (25 μg/ml)	687 ± 157.8	$6.5X10^{-5}$
Trypsin treatment of parasites (100μg/ml) (binding inhibition control)	17.6 ± 16.1	
no carbohydrate added (binding control)	313.6 ± 133	0.00194

Table 3.3. Inhibition of cytoadhesion of selected 3D7A to the CHO cell surface by incubation of parasites in the presence of different carbohydrates. Experiments conduced in duplicate on 3 separate occasions, N = 3, ± = standard deviation, (P= value refers to the statistical significance of the level of adhesion to CHO cells, as compared to the trypsin treated negative control, $P< 0.05$ = statistical significance)

3.2.5 Experiments to identify the CHO cell adhesion receptor by inhibition of 3D7A selected cytoadhesion by enzymatic treatment of CHO cells

To complement inhibition experiments using carbohydrates, experiments testing the binding of infected erythrocytes to CHO cells were also conducted after enzymatic treatment of CHO cells. To inhibit binding by chondroitin sulphate, 1 x 10^5 CHO cells were incubated in 1U/ml chondroitinase ABC (which digests chondroitin sulphate A, B and C) for 1 hour at 37°C. The cells were then allowed to attach to a 30 mm plastic petri

dish before being incubated with CHO cell-selected 3D7A parasites. However this treatment failed to inhibit adhesion of infected erythrocytes to CHO cells (Table 3.4, P = 0.002). CHO cells were also treated with heparinase-1 to remove heparan sulphate from the surface of CHO cells, which also failed to inhibit subsequent adhesion of selected 3D7A infected erythrocytes 3D7A (Table 3.4, P = 0.0018). In order to confirm that hyaluronic acid was not involved in adhesion CHO cells were also treated with hyaluronidase. Consistent with the GAG inhibition assay, treatment of cells with this proteolytic enzyme failed to inhibit adhesion (Table 3.4, P = 0.0016). Cells treated with neuraminidase were also used in an attempt to inhibit binding, without any effect on adhesion of infected erythrocytes (Table 3.4, P = 0.00010).

As a positive control for inhibition, some parasites were treated with l00 μg/ml trypsin for 10 minutes and prepared for binding as above The trypsin treatment inhibited binding of selected 3D7A to CHO cells by > 95% (Table 3.4, P = 0.0019). These results support those of the previous section, that selected 3D7A is adhering to the CHO cell surface via an as yet undescribed adhesion receptor expressed at the surface of CHO cells.

Table 3.4 Inhibition of binding of selected 3D7A parasites by enzymatic digestion

ENZYME TREATMENT	NO. OF PARASITES BOUND PER 1000 CHO CELLS	T TEST (P=)
Chondroitinase ABC (1U/ml)	515 ± 243.8	0.00203
Heparinase 1(1U/ml)	450.5 ± 207.2	0.00182
Hyaluronidase (1U/ml)	488.6 ± 219.9	0.001635
Neuraminidase (1U/ml)	636 ± 159.6	0.000102
Trypsin treatment of parasites (100µg/ml)	17.6 ± 16.1	
Control (No enzyme added)	313.6 ± 144.5	0.00195

Table 3.4 CHO cells treated with various enzymes to inhibit infected erythrocyte adhesion. Cells transfered into 30 mm culture dishes and allowed to adhere, selected 3D7A allowed to adhere to the CHO cell surface. Experiment conducted in duplicate N = 3, ± = standard deviation (P= value refers to the statistical significance of the level of adhesion to CHO cells, as compared to the trypsin treated negative control, $P < 0.05$ = statistical significance)

3.2.6 Binding to a CHO cell mutant, CHO-745, defective in glycosaminoglycan biosynthesis

Another approach to investigating the binding properties of the CHO-selected 3D7A involved incubating the selected parasites with a CHO cell line, CHO-745. CHO -745 is deficient in xylosyltransferase activity, an enzyme which is essential for the biosynthesis of glycosaminoglycans (Esko *et al.*,1985). CHO-745 does not express CSA, CSC, hyaluronic acid or heparan sulphate (Esko *et al.,* 1985; Rogerson *et al.,* 1995; Scherf *et al.,* 1998). The CHO-745 cell line was prepared in a manner identical to that used for preparing standard CHO cells (which were used as a control). The binding of selected and unselected 3D7A parasites to these two cell lines was then compared. The selected 3D7A parasites bound to the CHO-745 mutant cell line and the CHO cell line at similar levels (P = 0.1197), supporting the conclusion derived from earlier experiments, that binding of selected 3D7A to CHO cells is not mediated by CSA, CSC, Hyaluronic acid or heparan sulphate (Table 3.5).

Table 3.5 Binding of selected 3D7A to CHO 745 mutant CHO cell line

CHO CELL LINE	NO OF PARASITES BOUND PER 1000 CHO CELLS	T TEST (P=)
CHO	516 ± 164.1	0.0001869
CHO-745	714.4 ± 546.6	0.0011426
CHO (trypsin treatment of parasites 100μg/ml)	12.8 ± 13.3	

Table 3.5 30 mm culture dishes seeded with CHO and CHO -745 cells, grown until semi-confluent. 3D7A parasite used for cytoadhesion to CHO cells with a typsin treated CHO cell line as control. Adhesion assays conducted in duplicate, N = 3, ± = standard deviation. (P= value refers to the statistical significance of the level of adhesion to CHO cells, as compared to the trypsin treated negative control, $P < 0.05$ = statistical significance)

3.3 Conclusions

The evidence from this chapter suggests that 3D7A, after 8 successive rounds of selection, can be selected for significant levels of adhesion to the surface of CHO cells. Protease digestion of the infected erythrocyte with trypsin abolishes adhesion, which suggests that the adhesion is being mediated by a trypsin sensitive parasite derived adhesion receptor. PfEMP-1 mediated adhesion is trypsin sensitive. It is therefore possible that the this adhesive phenotype is mediated by a member of the PfEMP-1 family.

The adhesion was not inhibited by the addition of soluble CSA to parasites prior to binding, suggesting that this adhesion is not mediated by CSA. This contention is

supported by the observation that enzymatic digestion of CHO cells with chondroitinase ABC also failed to have any effect on adhesion or selected 3D7A to the CHO cell surface. The adhesion assays with the chondroitin sulphate A deficient CHO-745 cell line provide further evidence that CSA is not involved in this cytoadhesion process.

Another GAG investigated as an adhesive receptor was chondroitin sulphate C. Neither inhibition by adding this GAG to the binding experiment nor the treatment of CHO cells with chondroitinase ABC inhibited binding. The selected parasites also adhered to the CSC deficient CHO-745 cells. Chondroitin sulphate C can thus be eliminated as the adhesive receptor mediating this interaction between selected 3D7A and the CHO cell surface.

The possibility that the rosetting receptor heparan sulphate is a cytoadherence receptor was explored. Enzymatic digestion of CHO cells with heparinase –1 and the addition of heparan sulphate to the binding medium failed to inhibit binding. The ability of selected 3D7A to adhere to the CHO-745 mutant provides compounding evidence that heparan sulphate is not the adhesive receptor mediating this adhesive phenotype. Similar results were obtained with the novel placental adhesion receptor hyaluronic acid and neuraminic acid. Neither of the inhibition experiments inhibited binding, thus eliminating these cell surface molecules as adhesion receptors mediating the adhesion of selected 3D7A and the CHO cell surface. Collectively these results suggest that the carbohydrates tested are not involved in this adhesive phenotype and that another cytoadherence receptor expressed at the surface of the CHO cells mediates must be involved.

Chapter 4

Characterisation of PfEMP-1/*var* gene transcription in unselected 3D7A cultures

4.1 Introduction

Although *var* genes exhibit a great deal of sequence diversity, PCR and RT-PCR assays have been successfully used to amplify different members of the gene family to analyse sequence diversity (Kyes *et al.,* 1997; Ward *et al.,* 1999), transcriptional regulation (Fischer *et al.,* 1997; Chen *et al.,* 1998 a; Scherf *et al.*, 1998) and protein function (Chen *et al.,* 1998 a; Rowe *et al.,* 1997; Buffet *et al.,* 1999; Reeder *et al.,* 1999; Smith *et al.,* 2000 a). These amplification reactions employ primers based on conserved sequence motifs within DBL domains.

Based on previously published findings, it was predicted that an unselected parasite culture would transcribe some or even all of the *var* genes in its genome. On the other hand, parasite cultures selected for adhesion to a particular receptor, seem to transcribe predominantly a single *var* gene, presumed to be involved in the adhesion process (Scherf *et al.,*1998; Chen *et al.,* 1998 a). To compare the *var* transcripts in CHO-cell adhesive 3D7A with those in unselected cultures, degenerate PCR primers were designed based on the sequences described by Su *et al.* (1995). A RT-PCR assay was designed to investigate the number of *var* genes transcribed by the unselected 3D7A clone.

4.2 Results

4.2.1 PCR of *var* gene sequences from 3D7A genomic DNA

Genomic DNA from 3D7A for PCR was prepared by standard methods. The DNA concentration was roughly quantified on an agarose gel (Fig. 4.1). Primers mod15 and mod13 (Kyes *et al.,* 1997; Ward *et al.,* 1999) were used to assay for the integrity of the isolated genomic DNA by comparing the amplification of genomic DNA against cloned DNA (data not shown).

Figure 4.1 Quantification of genomic DNA isolated from trophozoite/schizont stage 3D7A parasites for PCR

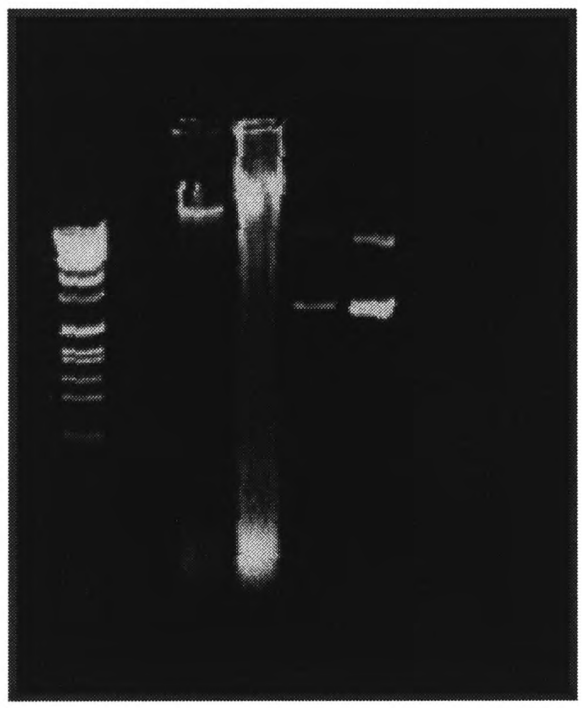

Figure 4.1 DNA was extracted from 3D7A parasites by saponin lysis and proteinase K treatment, run on a 1.2% agarose gel for quantification. 1. molecular weight marker VII, 2. Blank, 3. 3D7A DNA 125 ng/ml (4 µl), 4. 3D7A DNA 0.5 µg/ml (4 µl), 5. pGem (plasmid DNA) 200 ng and 6. pGem. 500 ng

Degenerate primers for PCR were designed to amplify as many members of the *var* gene family as posible. Five primers were designed to conserved regions within DBL-α, DBL-δ and exon 2 (Fig. 4.2 and Table 4.1). The PCR conditions were optimised using an annealing temperature gradient and by adjusting the extension time. It was found that a reduction the denaturation temperature from 94°C, used in many *Plasmodium falciparum* PCR protocols (Kyes *et al.,* 1997) to 93°C improved the efficiency of the

PCR reaction. Due to the degeneracy of the primers and the high AT content of the *P. falciparum* genome the annealing temperature used was also relatively low (51°C) (Gardener *et al.*, 1998; Bowman *et al.*, 1999).

Table 4.1 Primer sequences for the amplification of var gene sequences.

PRIMER NAME	PRIMER SEQUENCE 5'TO 3'	DIRECTION
Mod 13	GCACTCGAGTTATTCTTYTY TTTGGTTATCTATCCA	REVERSE
Mod 15	CGAGGATCCCCATATAGACG ATTACATSTATG	FORWARD
*Var*3	GGTGCGGCCGCWGGWACAT AWATATCATTWATRTC	REVERSE
Var 4	GGTTCTAAWACWACTTCWA TTAAWGTTTTATATTTW	REVERSE
Var 5	GGTGCGGCCGCTARTCGYCT CCTGGGTGGSAYAC	REVERSE
Var 6	TCGCGGATCCACCCAGGAGR CGACGAYTATAC	FORWARD
Var 8	TCGCGGATCCACGMAGTTTT GCRGAYATWGG	FORWARD

Table 4.1 primer sequences used to amplify *var* gene sequences from 3D7A. Reverse primer Mod 13 contains a restriction site for the enzyme Xho-I (in blue lettering). The Forward primer Mod 15 has a restriction site for the enzyme Bam HI (in pink lettering). All other primers were designed without restriction sites.

REDUNDANCIES

M=A/C, R=A/G, W=A/T, S=C/G, Y=C/T, K=G/T, V=A/C/G, H= A/C/G, D=A/G/T, B= C/G/T, N=A/C/G/T

Figure 4.2 Var gene schematic

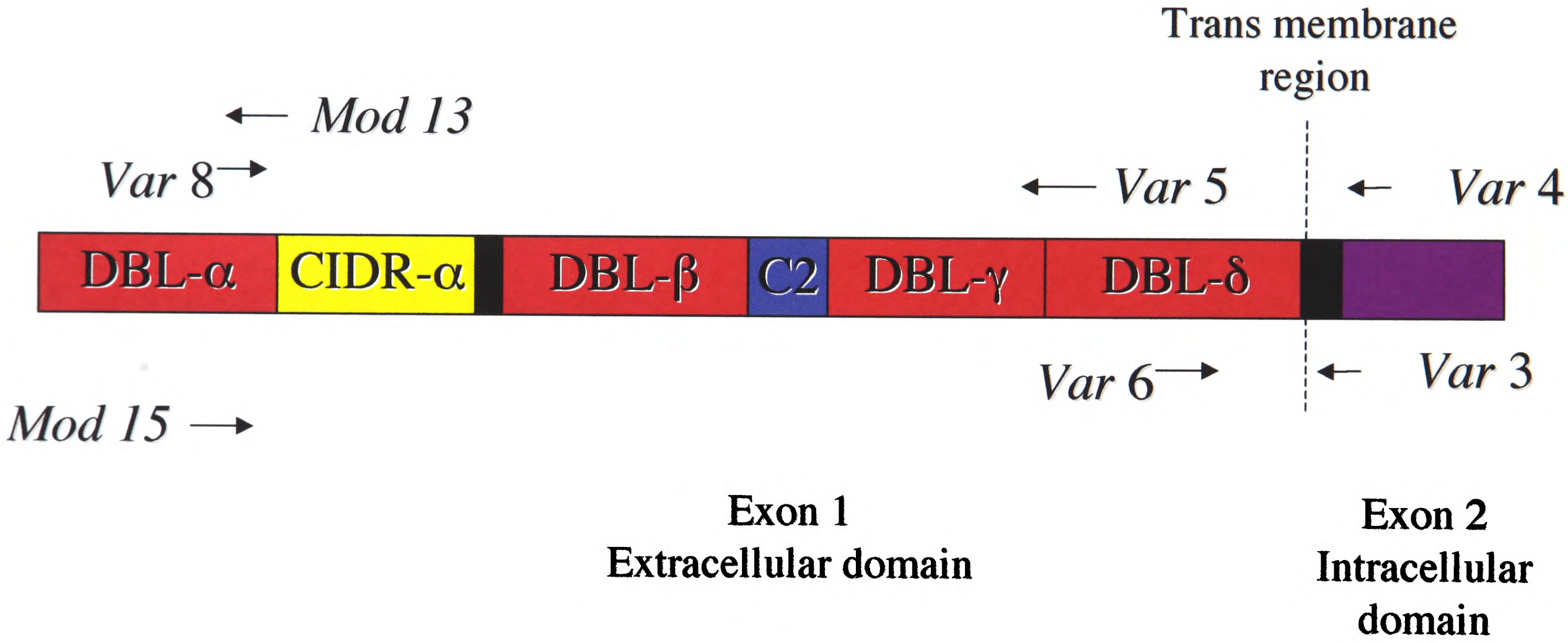

Figure 4.2. Schematic of *var* gene with DBL (Red) CIDR (yellow)and C2 (Blue) domains. The exon 2 region is shaded purple. Regions of *var* genes to which primers anneal are indicated. Arrows indicate direction of primers.

Table 4.2 . PCR products amplified from genomic DNA using different primer pairs and the expected size of these PCR products.

PRIMER PAIR	OBSERVED BAND SIZE (KB)	EXPECTED BAND SIZE (KB)
Mod15- *Var* 4		4-9
Mod15- *Var* 3		4-9
Var r8- *Var* 4	~ 6.1	4-9
Var 8-v *Var* 3	~ 6.1	4-9
Var 6- *Var* 4	~ 3.6	3-4
Var 6- *Var* 3	~ 3.6	3-4
Var 5-Mod15	~ 2.4	2-3
Var 5- *Var* 8	~ 2.4	2-3
Mod 15-mod13	0.6	0.7

Table 4.2 The observed band size of PCR products amplified from genomic DNA by specific primer pairs.The expected band size was calculated by aligning these primer pairs against *var* gene sequences submitted to the NCBI genebank database .

Using these degenerate primers several PCR products were amplified, many of which fell within the expected size range (Fig. 4.3, Table 4.2). The expected size of PCR products was calculated by aligning the primers used against published *var* gene sequences using the Macvector™ program. The distance between the primer pairs was used to calculate the expected band size for each primer combination. *var 8-var 4* and *var 8-var 3* produced a PCR band of 6.1 kb (within the expected range of 4-9 kb, Table 4.2). The primer combination *var 6-var 4* and *var 6-var 3* produced a PCR product 3.6 kb which is within the expected range of 3-4 kb. The primer combination *var 5*-mod 15 and *var 5-var 8* produced a PCR band 2.4 kb (within the expected size range 2-3 (Table 4.2). The primer pair mod 15-mod 13 produced a PCR product of 600bp, again the size expected. (Table 4.2). Having thus determined that the primers chosen for PCR amplification produced amplified fragments of expected size, they were then used in the RT-PCR experiments.

Figure 4.3 PCR amplification of var genes from 3D7A DNA

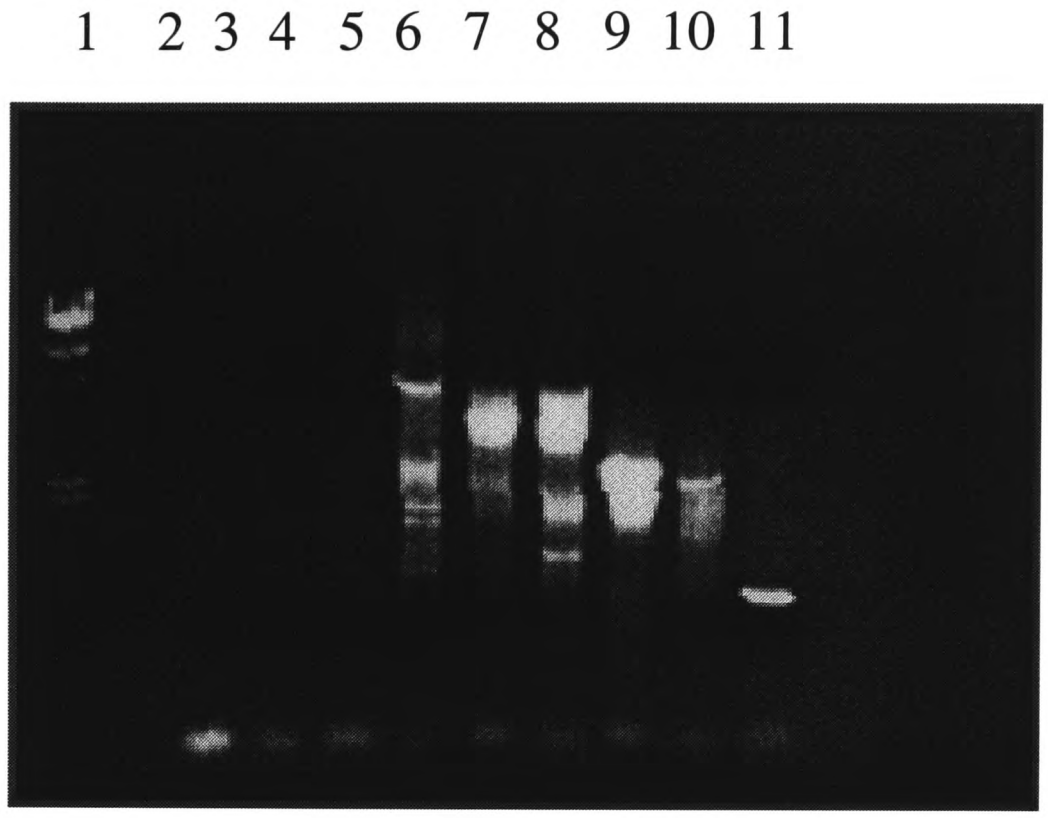

Figure 4.3 PCR amplification of *var* gene sequences from genomic DNA using a variety of primer combinations. 1. Molecular weight Marker II, 2. Blank 3. mod 15- *var* 4, 4. Mod 15- *var* 3, 5. *var* 8- *var* 4, 6. *var* 8-*var* 3, 7. *var* 6- *var* 4, 8. *var* 6- *var* 3, 9. *var* 5-mod15, 10. *var* 5- *var* 8, 11. mod 15-mod 13

4.2.2 RT-PCR of RNA from unselected 3D7A

Parasites prepared for RT-PCR were cultured using essentially the same protocol for producing DNA, with the two variations being that parasites were cultured as bulk cultures of 6-8 large flasks and then synchronised using 5% sorbitol. Cultures were then harvested at 10% parasitaemia when approximately 95% of the parasites in culture were at early developmental stages 6-9 hours post invasion. RNA was isolated using an RNA isolator™ kit, a nucleic acid extraction protocol employing phenol and guanidinium thiocyanate . An aliquot of the RNA was used to quantify roughly the amount of RNA on a gel (Fig. 4.4). RNA isolated in this way can be contaminated with DNA, although this problem can be overcome by treatment of the RNA sample with RNAse free DNAse. An aliquot of DNase treated RNA was used in a reverse transcriptase reaction. The remainder was stored at -70°C.

Figure 4.4 Quantification of total RNA isolated by RNA isolator from 6 flasks of ring stage parasites.

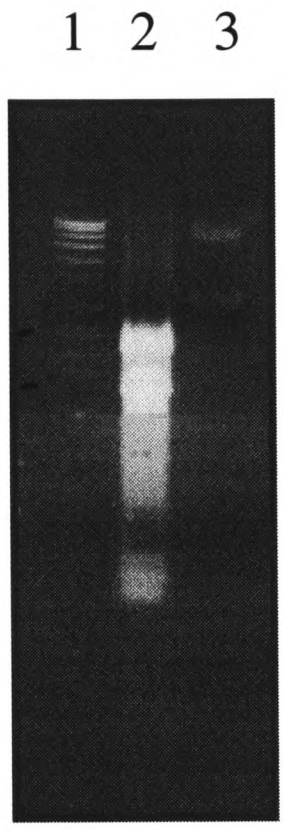

Figure 4.4 RNA isolated from 6 flasks of parasites using RNA isolator™ 1. molecular weight marker VII, 2. RNA 200 ng/ml (5 µl), 3 . pGem 200 ng

Since var genes are large genes, encoding up to 4000 amino acids and up to ~13kb in size, reverse transcribing mRNA of this length efficiently is extremely difficult. In order to polymerise the longest reverse transcripts possible, long range RT-PCR using Expand™ reverse transcriptase was used. This employs a modified enzyme and optimised buffers. Furthermore it was found that the small scale cultures initially used to optimise the RT-PCR using the mod 13 and mod 15 primers (yielding a 700bp RT-PCR product) were insufficient to provide enough high quality intact RNA to generate longer transcripts (data not shown). Therefore the number of flasks used was increased to 6-8. RNA was then reverse transcribed from these large scale cultures to produce cDNA.

The same primer pairs and conditions used in the amplification of genomic DNA were used in the amplification of cDNA (Fig. 4.5, Table 4.3). The primers were aligned to published sequences (minus the 1 kb intron separating the genomic DNA encoding the external domains from the transmembrane region) to calculate the expected band size.

Primer combination *var 8-var 3* produced a PCR band of 2.4 kb (this was not within the expected range of 4-9 kb, Table 4.2). This may have been due to mis-priming of either of the PCR primers. The primer combination *var 5*-mod 15 and *var 5-var 8* produced a PCR band 2.4 kb (within the expected size range 2-3 kb, Table 4.3). The primer pair mod 15-mod 13 produced a PCR product of 600 bp, again the size expected. The primer combination *var 6-var 3* produced a PCR product 2.9 kb (within the expected range 2-3 kb, Table 4.3). When PCR amplifying across the single *var* gene intron which is spliced out of the m-RNA sequence, the PCR band appears, as expected, approximately 1 kb shorter than the identical reaction performed on genomic DNA (Fig. 4.6). As a control for the presence of contaminating DNA, PCR of RNA not previously reverse transcribed failed to amplify the product. These results confirm that the PCR band present in the cDNA reaction was due to amplification of reverse transcribed RNA and not contaminating DNA (Fig. 4.6 lane 5 and Fig. 4.7 lane 6).

Table 4.3 Table of RT-PCR products amplified from cDNA using different primer pairs

PRIMER PAIR	OBSERVED SIZE (KB)	EXPECTED SIZE (KB)
Mod 15-mod 13	0.6	0.7
Var 5-*Var* 8	2.4	2-3
Var 6-*Var* 3	2.9	2-3
Var 8-*Var* 3	2.4	3-8
Mod 15-mod 13	-	0.7
Mod 15-mod 13	0.6	0.7

Table 4.3 Observed band sizes of PCR products amplified form cDNA reverse transcribed from RNA extracted from unselected 3D7A. Expected band sizes calculated by alignment of *var* gene sequences submitted to the NCBI Genebank database.

Figure 4.5 RT-PCR of total RNA isolated from ring stage parasites.

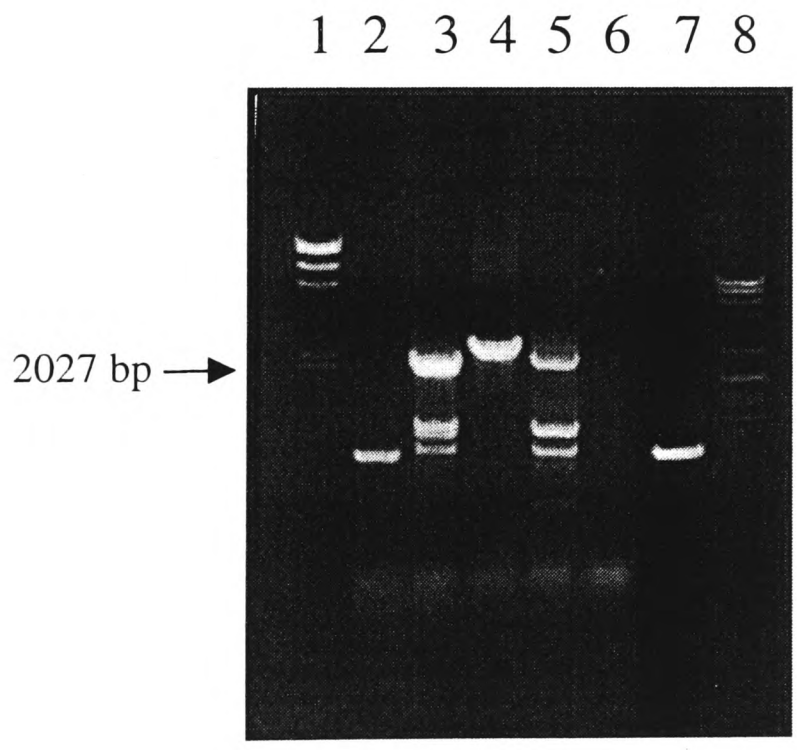

Figure 4.5 PCR amplification of *var* gene sequences from cDNA using a variety of primer combinations, 1. molecular weight marker II, 2. mod 15-mod 13, 3. *var* 5- *var* 8, 4. *var* 6- *var* 3, 5. *var* 8- *var* 3, 6. mod 15-mod 13, 7. mod 15-mod 13 amplified from DNA and 8. molecular weight marker VII.

Figure 4.6

PCR amplification of genomic DNA and cDNA across the var gene intron using primer var 6- var 3.

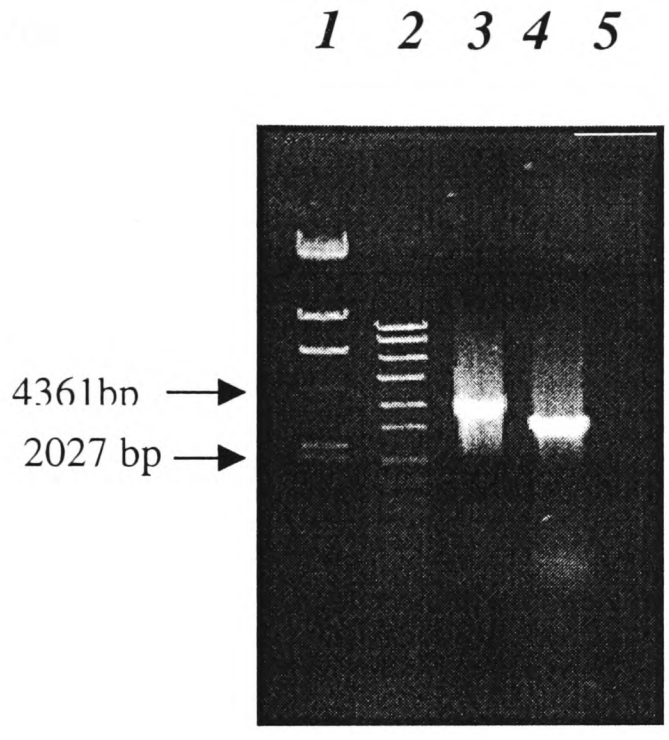

Figure 4.6 PCR amplification using primers *var* 3 and *var* 6 1. molecular weight marker II, 2. molecular weight marker VII, 3. DNA 3.6 kb PCR porduct, amplification across the *var* gene intron, 4. cDNA amplification, 2.9kb, no intron present thus giving a 0.7 kb smaller PCR product.

Figure 4.7 PCR amplification of the 5' end of var genes using primer var 8- var5

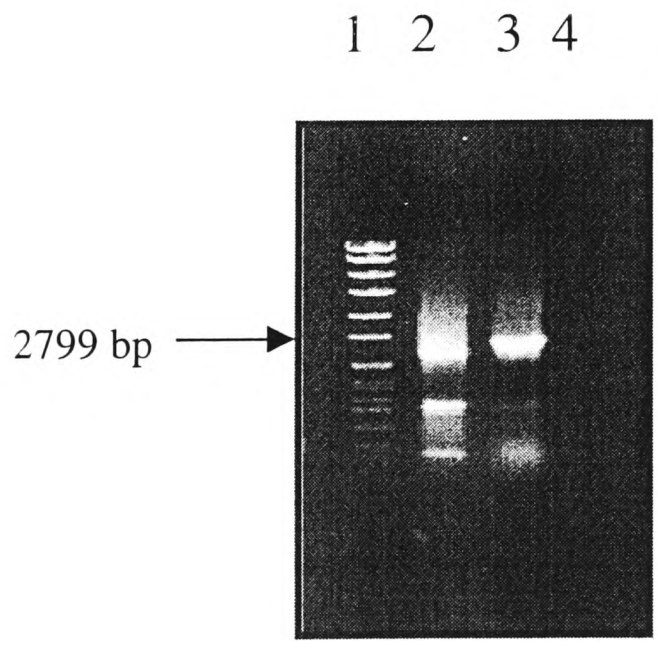

Figure 4.7 PCR amplification of DNA cDNA and RNA using primers *var* 5 and *var* 8, 1. molecular weight marker VII, 2. amplified DNA (2.4 kb PCR product) 3. cDNA (2.4 kb PCR product) and 4. RNA (not reverse transcribed). Note no amplification, i.e. no contaminating genomic DNA.in this preparation.

4.2.3 Cloning and sequencing transcribed *var* gene sequences from unselected 3D7A

Although a number of primers permitted amplification of products from both genomic DNA and cDNA templates, the primer combination *var 8-var 5* and *var 6-var 3* consistently produced strong PCR bands and these products together covered exon 1, (Figs. 4.4 - 7). The PCR products from these two reactions were excised from the gel, purified and where necessary concentrated by ethanol precipitation. PCR products were ligated into a TA cloning vector, transformed into competent *E .coli* cells and plated on to LB ampicillin plates. β-galactosidase dependent blue-white screening was used to select clones containing insert. White (insert positive) colonies were picked and first screened for insert by PCR using the vector primers T7 and M13R. (data not shown). PCR positive clones were screened by restriction digest with Xho-I (Fig. 4.8).

Figure 4.8
Xho-I digest of positive clones obtained after ligation and transformation of PCR products amplified from unselected 3D7A cDNA using primers var 8-var 5 primers.

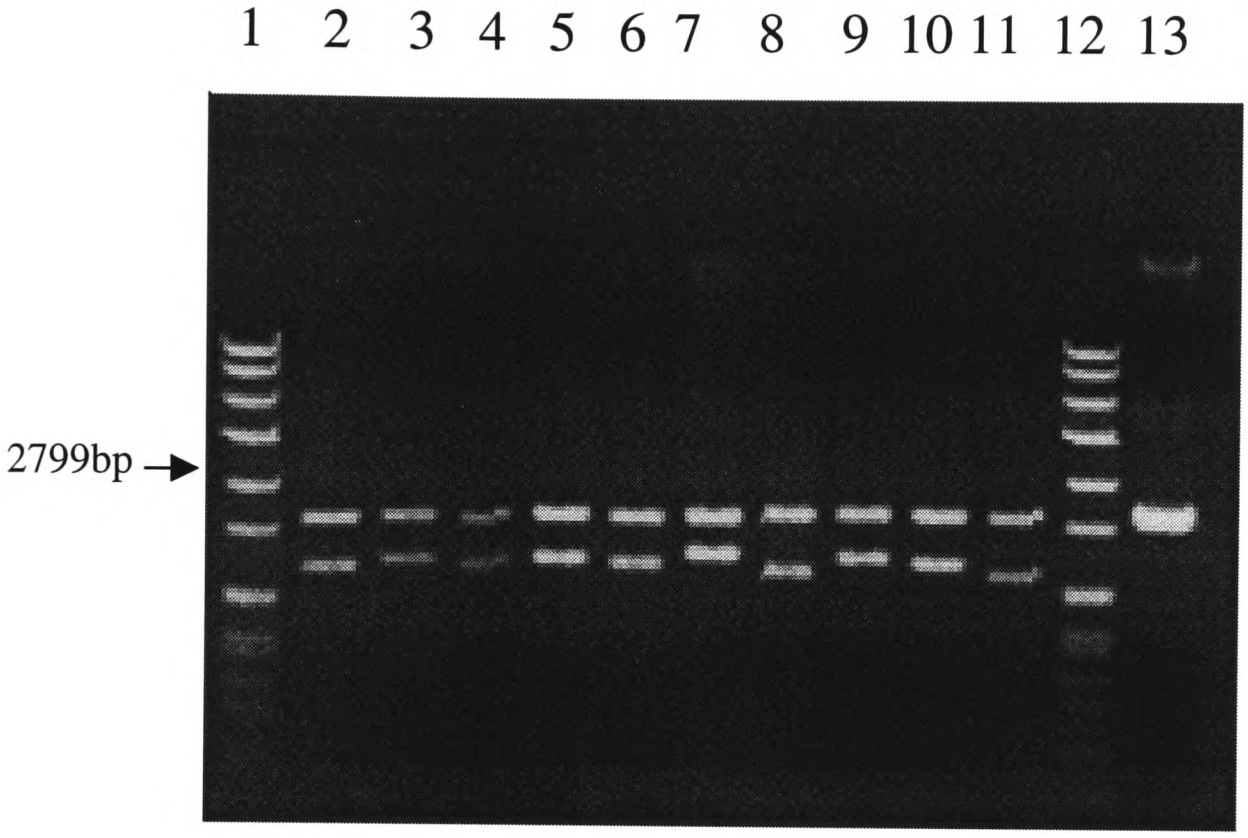

Figure 4.8 Restriction digest of products cloned from the amplification of *var* gene sequences from cDNA using *var* 8-*var* 5 primers. 1. molecular weight marker VII, 2-11. clones b1-b10, (2.2-2.3kb), 12. molecular weight marker VII, and 13. Uncut pGem

4.2.4 Sequencing of *var* gene clones

Positive clones were sequenced on an ABI automated sequencer, using T7 and M 13R sequencing primers. Initially 10 clones from PCR amplifications using *var* 5-*var* 8 and *var* 6-*var*3 primers were sequenced in both directions. The sequences were then translated into amino acid sequence using Macvector™. Sequencing revealed that 4 different clones from the 10 *var 6 –var 3* clones were obtained and 8 different clones from the 10 *var 5-var 8* clones were obtained (Figs 4.9a, 4.9b and 4.10a, 4.10b respectively). The sequences were aligned against *var* sequences submitted to NCBI sequence database and were found to be homologous to PfEMP-1 proteins, i.e. *var* 8-*var* 5 clones showed greatest homology with DBL-α and DBL-δ at the 5' end and 3' end of

each clone respectively. *Var* 3-*var* 6 PCR bands showed the greatest homology with DBL-δ and exon 2 of PfEMP-1 sequences.

In order to obtain a representative sample of the *var* gene transcripts transcribed by unselected 3D7A cultures more *var 5-var 8* clones were sequenced. A total of 217 clones were sequenced and a total of 27 different *var* sequences isolated (Fig. 4.11). Some sequences appeared more frequently than did others, although whether this represents a difference in abundance of transcripts or differences in efficiency of PCR primers to amplify different sequences is unclear.

4.2.5 Analysis of DBL-α *var* gene sequences

Analysis of the DBL-α sequence of these transcribed *var* genes revealed that the genes transcribed within the clone 3D7A were very diverse both at the nucleotide and the amino acid level. The diversity of *var* gene sequences can be illustrated by the very low sequence homology observed between the *var* gene clones 149 and 145, in which identity is only 19% at the amino acid level (Fig. 4.11). *Var* clones 211 and 177 appeared most similar, with nucleotide sequence homology of 84% and 78% amino acid identity (Fig. 4.11). Conserved sequences tended to appear in sequence blocks of between 10-20 amino acids. These sequence blocks appear periodically throughout the sequence. There are also several cysteine residues conserved between most sequences. There are also length polymorphisms located between the conserved sequence blocks (Fig.4.11).

4.2.6 Sequence comparison of 3D7 DBL-α sequences with DBL-α sequences cloned from parasites of diverse geographical origins

The 27 different transcribed *var* sequences cloned from the unselected 3D7A parasite clone were aligned with 20 geographically diverse *P. falciparum var* sequences obtained from the Genebank Database and an additional 22 Sudanese sequences obtained from parasites isolated in a single village (Ward *et al.,* 1999). The Sudanese sequences were cloned from parasite clones SD101, SD102, SD105, SD106, SD126 and SD128 which were cloned from six unrelated malaria patients in the village of Asar, between October and November 1989. Each clone has a distinct phenotype, genotype and molecular karyotype (Babiker *et al.,* 1991; Bayoumi *et al.,* 1993; Bayoumi *et al.,* 1994). *Var* genes were amplified from the genomic DNA of these clones by PCR using primers which anneal to the 5' end of the DBL-α domain, and then cloned and sequenced. (Ward *et al,.*1999).

This analysis revealed a surprising degree of sequence similarity between certain 3D7A sequences and sequences cloned from the Sudanese parasite SD105. 3D7A *var*-149 sequence is identical to the SD105 sequence SD105J at the nucleotide level. The 3D7A *var*-59 sequence and the SD105M sequence share 332 of the 335 nucleotides sequenced. Other sequences with similar amino acid sequences were observed. For example 3D7A *var*-37 and SD105L share 124 of 125 amino acids, 3D7A *var*-43 and SD105R share 128 of 131 amino acids and 3D7A *var* 3 and SD105F have 100 of 111 identical sequences. 9 of the differences in the latter comparison occur over a continuous stretch of sequence.

4.2.7 Mapping of *var* genes to 3D7A chromosomes sequenced by the Malaria Genome Project.

Alignment of the 3D7A transcribed sequences obtained in the current work against the 4 complete 3D7A *var* genes sequenced and published to date has permitted the mapping of

two of these transcribed genes to a particular chromosome. The amino acid sequence of the 4 *var* genes sequenced to data were aligned against the 27 *var* gene clones from the unselected 3D7A parasite culture using the MacVector program™. The transcribed 3D7A *var* clones 177 and 51 were mapped to the left and right arms of chromosome 3 respectively. Attempts were made to map the remaining 25 clones to specific contigs or sequence reads derived from the different chromosome sequencing projects; however this has been found to be much more difficult than expected due to the contamination of purified chromosomal DNA preparations used to generate chromosome specific clone banks with genomic DNA from neighbouring chromosomes (Bowman *et al.*, 1999). Therefore until each 'chromosome' is fully sequenced and more importantly annotated, further mapping of these sequences "*in silico*" is not possible.

Figure 4.9A Sequence alignment of 5' end of *var* 8-*var* 5 clones

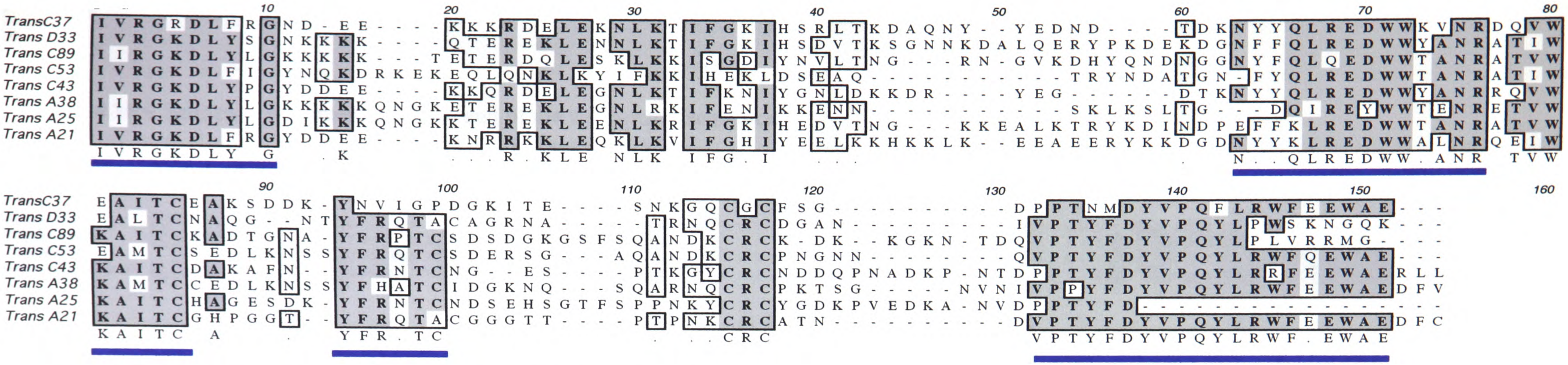

Figure 4.9B Sequence alignment of 3' end of *var* 8-*var* 5 clones

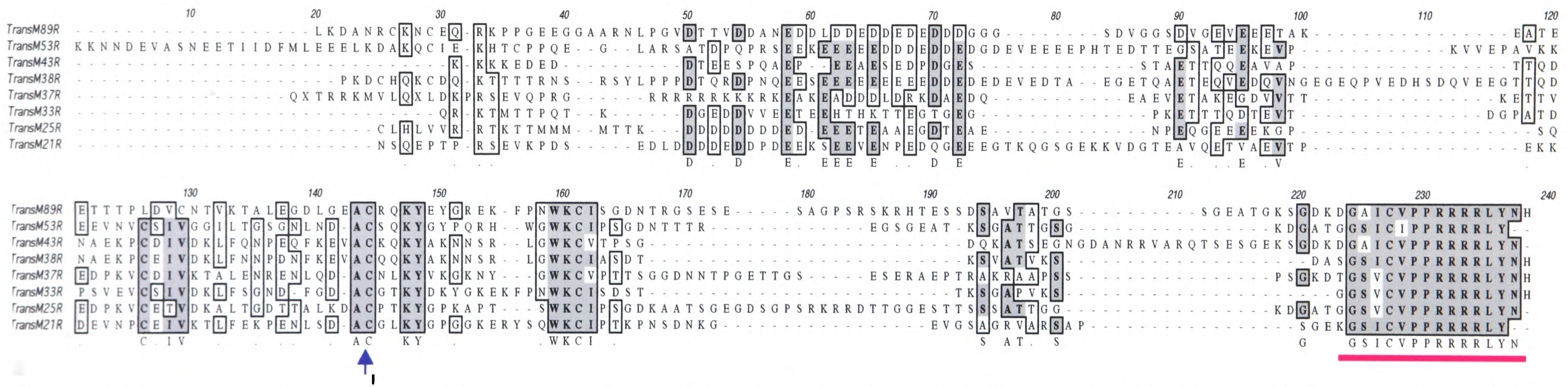

Figure 4.9 Alignment of cDNA clones amplified from unselected 3D7A parasite cultures. **4.9A.** 5' sequence of cDNA clones (primer sequence removed). Sequence identity with the 5' end of DBL-α domains submitted to NCBI Genegank. Regions of significant homology shaded in dark grey. Conserved sequence blocks underlined in blue. **4.9B** 3' end sequence of the same 8 cDNA clones (primer sequence removed). Sequence identity with the 5' end of DBL-δ domain of *var* genes submitted to the NCBI Gensbank database. Regions of significant sequence identity are shaded dark grey. Conserved sequence blocks underlined in pink. Blue arrow indicates start of DBL-δ sequence.

Figure 4.10A Sequence alignment of the 5' end of the *var* 6-*var* 3 sequences

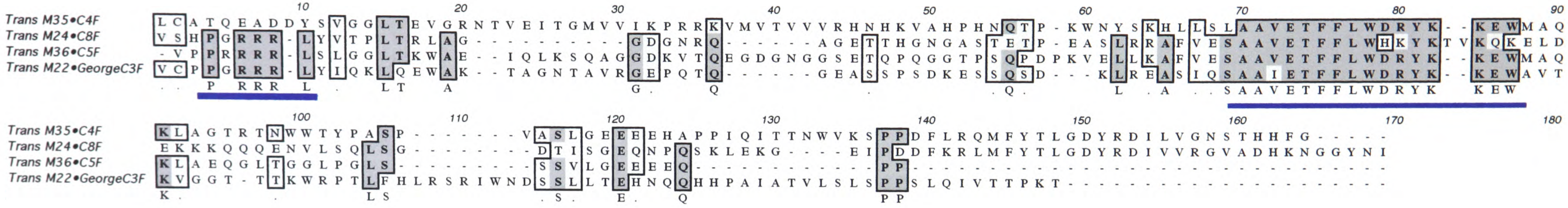

Figure 4.10B Sequence alignment of the 3' end of the *var* 6-*var* 3 sequences

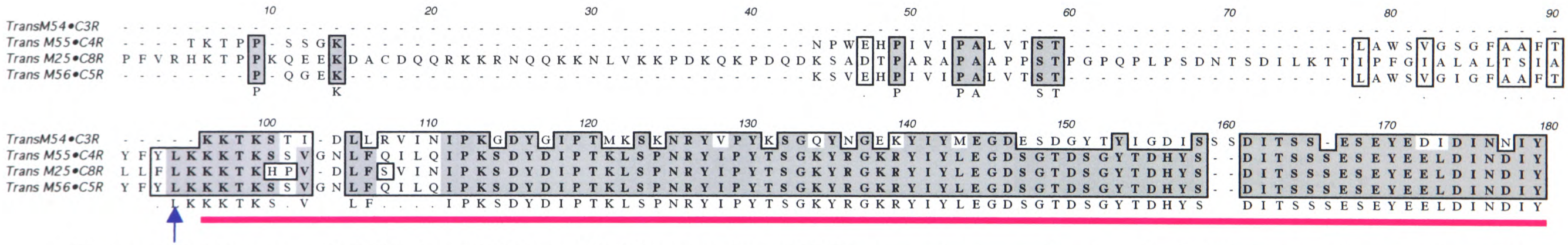

Figure 4.10 Alignment of cDNA clones PCR amplified from unselected 3D7A using primers *var* 6 and *var* 3. **4.10 A** sequence form the 5' end of this clone. Regions of significant homology are shaded dark grey . Gaps have been inserted to accommodate length polymorphisms. DBL-δ sequence motifs indicated by blue line. **4.10B** 3' end of the same set of clones, again regions of sequence homology shaded dark grey. Exon 2 sequence indicated by pink line. The blue arrow indicates the transmembrane region.

Figure 4.11 Alignment of the 5' end of *var* 3 *var* 8 clones.

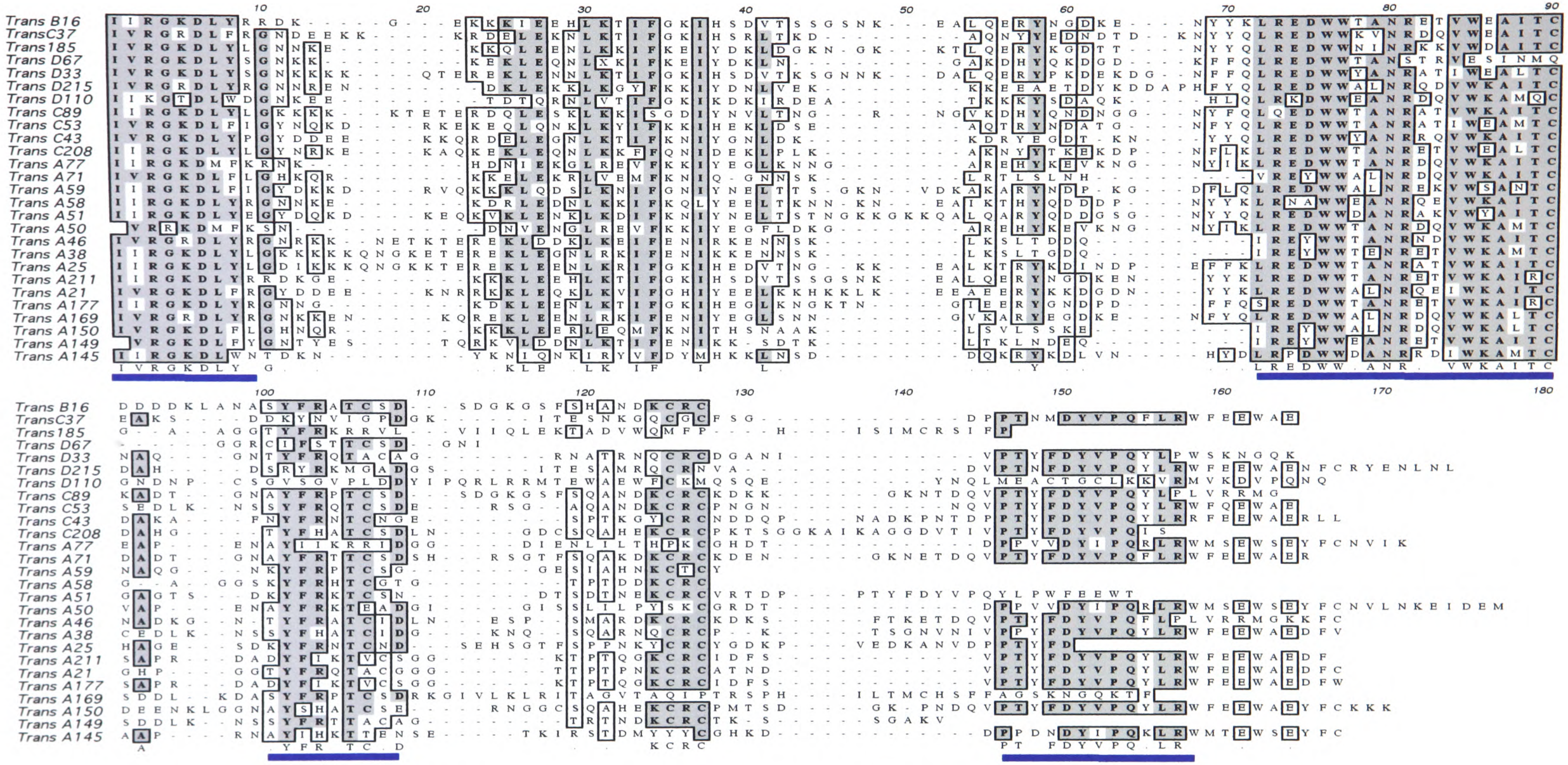

Figure 4.11 Alignment of the 5' DBL-α amino acid sequence of 27 cDNA clones amplified from unselected 3D7A.Primer sequence has been removed. Regions of significant homology have been shaded dark grey. Homology sequence blocks are indicated by blue line. Gaps have been inserted to accommodate length polymorphisms.

4.3 Conclusions

Twenty-seven different *var* gene sequences have been cloned from 3D7A ring stage cDNA. These clones contain a 5' DBL-α sequence at the 5' end of the sequence cloned and a DBL-δ sequence at the 3' end of the clone. These cloned gene fragments contain *var* gene sequence spanning the region between DBL-α and DBL-δ. These gene sequences were shorter than most sequences compiled from the Genebank sequence database, but are consistent with the size of the other *var* genes sequenced from the 3D7A genome. Twenty-seven different cDNA clones of an estimated 50 *var* genes have been cloned representing almost 3/5 of the *var* gene repertoire.

Sequence analysis shows that PfEMP-1 possesses great potential for sequence variation at both nucleotide and amino acid levels. It has been observed that the sequence homology between *var* sequences transcribed by the unselected 3D7A can be as low as 19% at the amino acid level. The sequence diversity is compounded by sequence length polymorphisms between *var* genes which create the potential for antigenic diversity by altering the protein structure. However an essential structural framework of the PfEMP-1 protein may be conferred by the conserved sequence blocks of between 10-20 amino acids observed at regular intervals throughout these sequences.

Sequence comparison of *var* genes from disparate geographical regions reveals that although the 3D7A parasite expresses a diverse set of *var* genes, sequence blocks within these *var* genes are not specific to this clone. *Var* gene sequence homology between 3D7A *var* genes and *var* genes cloned from Sudanese parasites in some cases ranged from 90-100% over stretch of 400 bp of sequence analysed. This exchange of *var* gene sequence between clones may occur via recombination of randomly mixing parasite clones in the field (Ward *et al.*, 1999).

The mapping of the transcribed *var* gene clones has permitted two sequences *var*-177 and *var*-51 to be mapped to the left and right arms of chromosome 3 respectively providing evidence that at least these chromosome 3 *var* genes are transcribed in an unselected parasite culture. Mapping of the remaining transcripts on to the as yet unassembled and unannotated *P. falciparum* genome sequence database has proved very difficult.

Chapter 5
Var gene transcribed by CHO cell adherent 3D7A

5.1 Introduction

Previous studies have shown that selection for a specific PfEMP-1 mediated adhesive phenotype also selects for the transcription of a predominant *var* gene sequence. This is the candidate sequence encoding the parasite protein mediating the adhesive phenotype (Chen *et al.*, 1998 a; Scherf *et al.*, 1998).

To identify the complete primary sequence of the PfEMP-1protein from 3D7A parasites involved in adhesion to the CHO cell surface a novel approach has been used. The 3D7A clone was chosen as the model clone for these experiments primarily because it is being sequenced by the *P. falciparum* genome project. Instead of using traditional "wet laboratory" methodology, after identifying a partial cDNA sequence from a RT-PCR clone it was decided that the full-length gene sequence be obtained "in silico" using sequence data being generated by the Malaria Genome Project.

5.2 Results

5.2.1 RT-PCR of 3D7A parasites selected for adhesion to CHO cells

Using PCR conditions employed in the amplification of the 27 *var* cDNA sequences, as developed in chapter 4 from unselected 3D7A cultures, primers *var* 5 and *var* 8 were used to amplify cDNA reverse transcribed from 3D7A selected for adherence to CHO cells. Others have demonstrated that selection for a PfEMP-1 mediated adhesive phenotype selects for a parasite population transcribing one predominant *var* gene transcript, the transcript that is subsequently translated into protein and expressed at the infected erythrocyte surface (Chen *et al.,* 1998 a; Scherf *et al,* 1998). Chen *et al.* (1998 a) demonstrated that recombinant fusion protein expression of the DBL-α domain sequence could be successfully used to block rosetting in a sequence specific manner. Scherf *et al.* (1998) further demonstrated that transfection of CHO cells with partial cDNA sequences corresponding to DBL-γ domain from the selected transcript supported adhesion in this cell line.

RT-PCR products from the CHO selected 3D7A were run on an agarose gel (Fig. 5.1). A single band was seen which was approximately 1.0kb (Fig. 5.1). The PCR product was 1.4 kb shorter than the 2.4 kb PCR product amplified from unselected 3D7A (Chapter 4). Thus the selection of 3D7A had apparently resulted in the selection for a sub-population of parasites transcribing *var* genes distinguishable by size from those sequences previously cloned from unselected 3D7A.

Figure 5.1 RT-PCR of 3D7A selected for increased adhesion to CHO cells

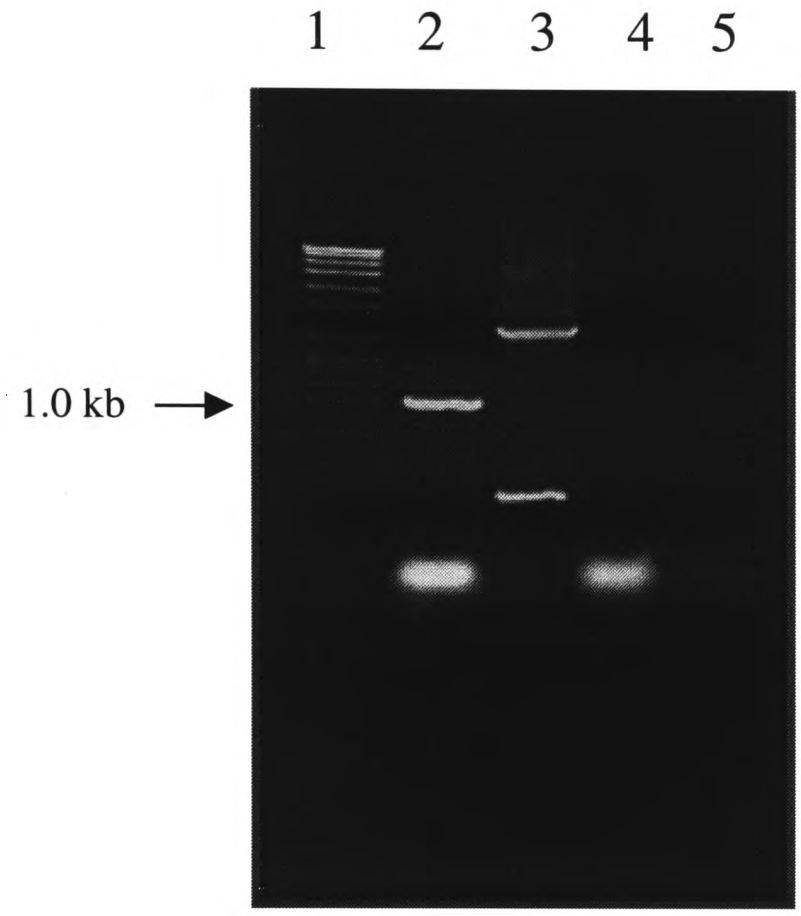

Figure 5.1 RT-PCR of parasites selected for adhesion to CHO cells. 1. Molecular weight marker VII, 2. 1.0kb PCR product amplified from cDNA , 3. 2.4 PCR product amplified from DNA (control), 4 . RNA(not reverse transcribed), 5. H_2O

5.2.2 Cloning & sequencing of RT-PCR products amplified from CHO cell selected 3D7A

The 1.0 kb PCR product was excised from the gel, purified, ligated into a TA cloning vector and transformed into competent cells, which were plated on LB ampicillin plates and grown over night. β-galactosidase-dependent blue/white screening was used to select colonies with insert. 10 insert positive colonies were picked and screened by PCR amplification using primers T7 and M13, and by restriction digestion with the enzyme Xho-I (data not shown).

10 insert positive colonies were sequenced in both directions using primers T7 and M13R. The sequences were translated and aligned using the MacVector™ program. One clone failed to sequence but 8/9 clones contained an identical sequence. This sequence was different from any of the 27 sequences originally cloned using these primers to amplify cDNA isolated from unselected 37DA (Fig. 5.2) clone. The cloning and sequencing of the PCR product was repeated once more with identical results i.e. 9 of 9 clones sequenced contained the one unique *var* gene sequence. These sequencing results indicate that selection for increased adhesion to the CHO cell surface (which is mediated by a parasite derived trypsin sensitive protein) also selects for the transcription of a predominant *var* gene sequence. Interestingly this predominant *var* transcript amplified from selected 3D7A was not identified as a *var* product in cDNA amplified from unselected 3D7A. The translation of the DNA sequence of the 1.0kb PCR product is shown in figure 5.3 . The cloned sequence was subsequently named *var*-CHO.

Figure 5.2 amino acid sequence of the var-CHO cDNA clone

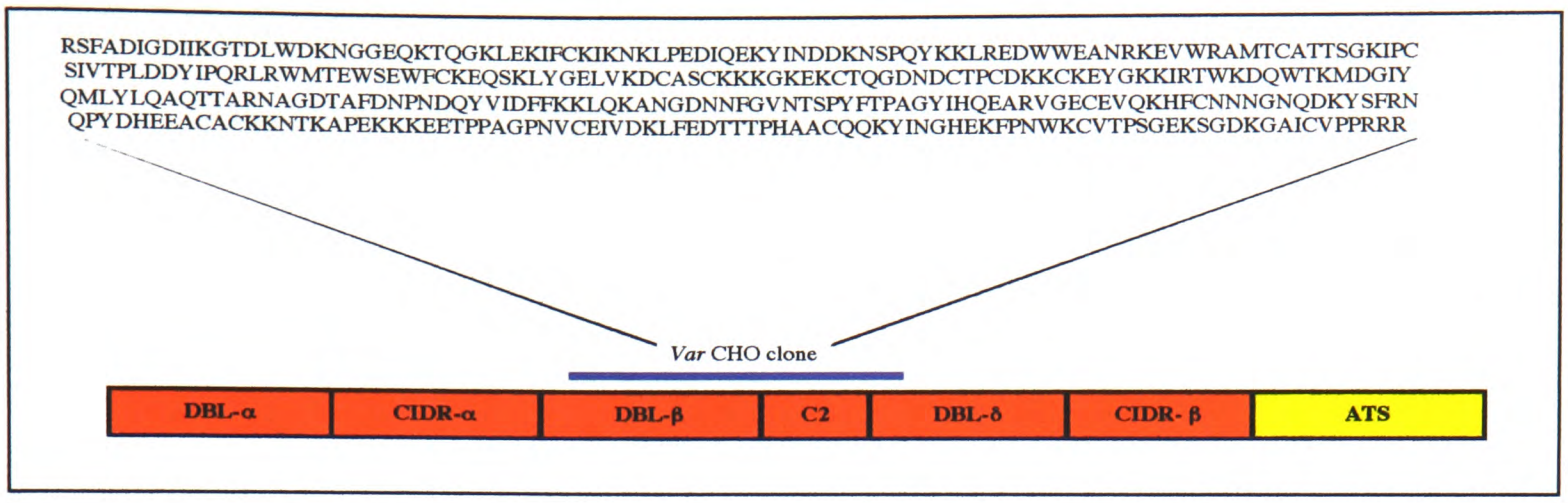

Figure 5.2 Amino acid sequence of the var CHO cDNA clone superimposed on to a schematic of the exon-one of the var-CHO gene. Blue line indicates position of cDNA clone (not to scale).

The cloned sequence obtained exhibited homology to both the DBL-β sequence at the 5' end and DBL-δ at the 3' end, when compared to *var* genes submitted to the NCBI Genebank database. This implies that the relatively short sequence amplified actually results from the mis-priming of the *var* 8, 5' primer to the ARSFADIGII sequence motif within the DBL-β domain. This motif is identical to the ARSFADIGII motif observed in all DBL-α domain sequences on which the *var* 8 primer was originally based. The smaller 1.0 kb fragment can be occasionally observed as a faint band in some RT-PCR amplifications of unselected 3D7A parasites (Fig. 4.7 chapter 4). However this relatively small band was not cloned from that RT-PCR amplification because at that time it was considered to be too small to have been derived from the PfEMP-1 target region to be amplified. It was thought that such bands probably represented amplification of non-*var* gene sequences, witch contain DBL type domains e.g. the EBA-175 gene.

5.2.3 Extending *var-CHO* sequence using genomics: accessing sequence data from the Malaria Genome Project

3D7A is the parasite clone being sequenced by the Malaria Genome Project. This is a collaborative project being conducted by three research institutes. The project has been divided between these institutes and each is sequencing particular chromosomes. The Sanger Centre in Cambridge is sequencing chromosomes 1, 3, 4, 5, 6, 7, 8, 9, & 13, Genome Technology Centre in Stanford California is sequencing chromosome 12 and the TIGR Institute For Genome Research in Rockville Maryland is sequencing chromosomes 2,10, 11 and 14. It was possible to exploit the availability of the rapidly accumulating sequence data from these centres to compile the complete *var*-CHO gene sequence.

The primary technique in such "in silico" molecular genetics is the blast search. This involves the submission of a nucleotide sequence to an Internet based algorithm, which identifies, within a particular database, sequences of significant homology with the sequence submitted. The Sanger Centre sequence database was the only one in which sequences with significant homology to the *var*-CHO sequence were identified in the period 1998-99. Sequence homology of > 98% in overlapping sequence was considered as evidence that the sequence reads came from truly contiguous fragments. Sequence overlap was analysed by eye. Overlapping sequences with a significant degree of homology were aligned in the Assemblylign™ program for nucleic acid alignment. The overlapping sequence was then again analysed by eye. These contiguous sequence reads

were then assembled to produce a contig, a variable number of overlapping sequence reads. Contigs were then assembled using the MacVector™ program and translated to ensure that the amino acid sequence remained in frame. The 3' and 5' sequence reads from the contig were then re-submitted to the Sanger Centre *P. faciparum* genome database and sequences obtained were then re-analysed for their sequence homology to the sequences originally submitted. Sequences with significant sequence homology were then re-aligned to the first sequence contig in the Assemblylign™ program to try to extend the sequence. Sequences were then once again re-analysed by eye and if necessary, adjusted by hand where they were poorly aligned by the computer program. This process was repeated with constant translation of the sequence to check that the sequence remained in frame.

The *var*-CHO sequence was extended to the presumptive 5' start methionine of the PfEMP-1 protein. The start methionine was deduced by alignment of the contiguous sequence against full-length *var* gene clones previously submitted to the NCBI Genebank database. Using the Sanger Centre genome database data it was also possible to extend the *var*-CHO sequence by a further 3kb from the 3' end of the cloned *var*-CHO cDNA clone. Eighty sequence reads including the cloned *var*-CHO sequence were used to extend this sequence from 1.0 kb to a total of 6.2 kb (Fig. 5.3).

To date it has not been possible to extend the sequence any further 3' using the Sanger Centre database. One of the major problems that the malaria genome sequencing program has faced is that the organisation of the task has been done by assigning particular chromosomes to particular centres. Individual chromosomes were separated

on pulse field agarose gels. The bands were subsequently cut out and DNA was purified from the excised bands prior to shot gun sub-cloning. Unfortunately clean separation is difficult for chromosomes 5-8 (the "blob") and all individual chromosomal preparations are variably contaminated with DNA from other chromosomes. It was therefore possible that the sequence contig could be extended using sequence reads from TIGR or Stanford sequencing projects as these chromosome preparations may actually contain contaminating DNA from chromosomes being sequenced by the Sanger Centre. Attempts were then made to continue the extension of the sequence using reads available from the *P. falciparum* chromosome 12 data base at the Stanford Genome Technology Centre Stanford, University. Also a blast search for homologous sequences was conducted using the "TIGR" database. However analysis of homology matches on these databases produced no significant sequence identity with the contig alignment assembled. The lack of any significant sequence homology with sequence reads obtained from these chromosomes did however aid the mapping of this gene to a specific chromosome (see below). The complete extension of the 3' end of the *var*-CHO sequence to the end of the exon-1 sequence did not prove possible "in silico" at least at this stage of development of the Malaria Genome Project. A more conventional directed approach to obtain the complete sequence was therefore used.

Figure 5.3 Contiguous alignment of sequence reads

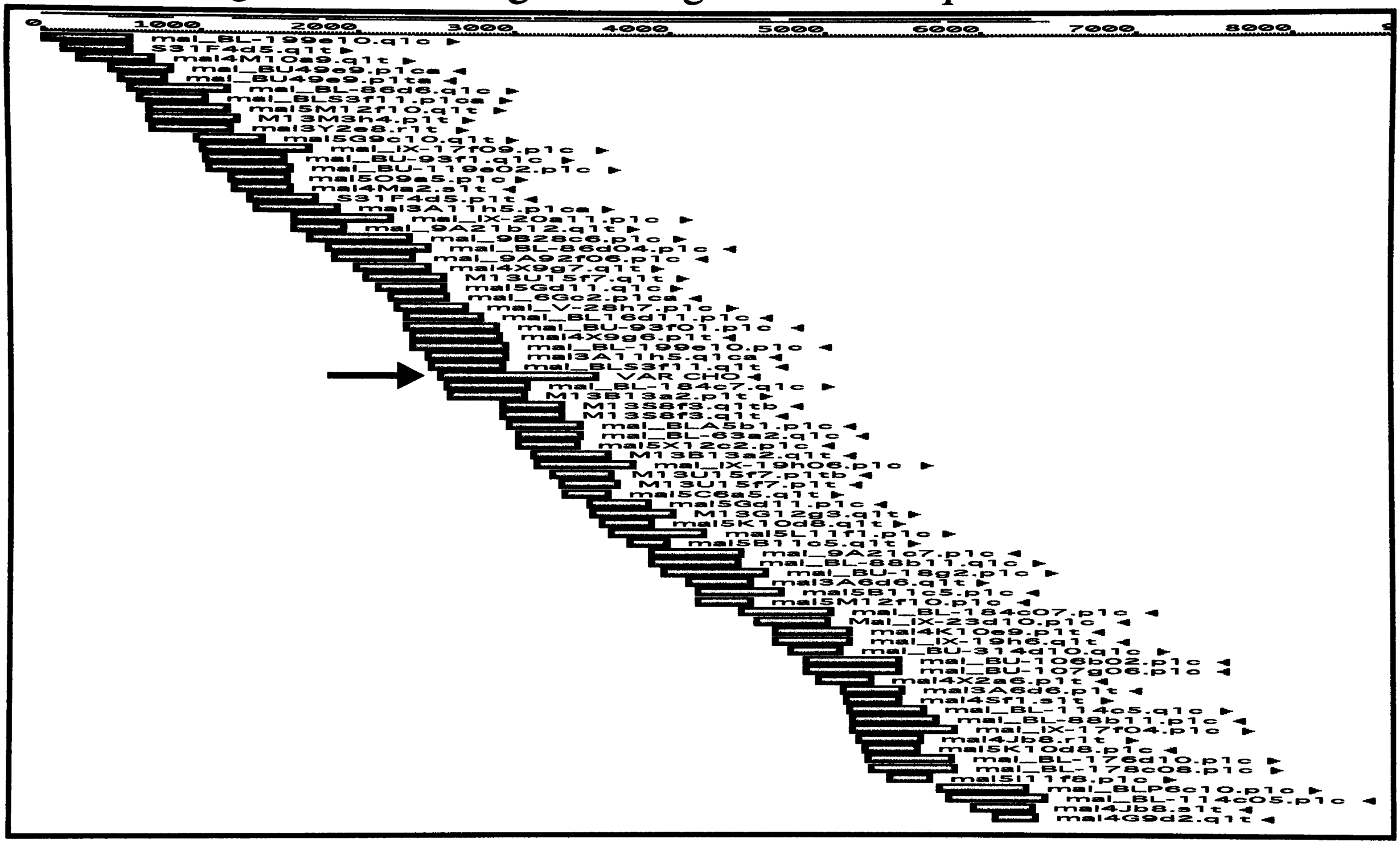

Figure 5.3 Sequence reads used to generate *var*-CHO contig. 80 sequence reads used including *var*-CHO clone (pink arrow). Codes refer to specific sequence reads used in assembling contig..Small black arrows illustrate clone orientation. length of contig 6619 bp.

5.2.4 Cloning the 3' end of exon1 of *var*-CHO

To extend the sequence 3' to the end of the exon-1 sequence, a forward primer (EX2) was designed to anneal to the 3' end of the *var*-CHO contig. The reverse primer used in this amplification was the *var* 3 primer, designed to anneal to a conserved region at the 5' end of exon 2. The primer pair Ex2 and *var* 3 were therefore used to PCR amplify the cDNA preparation used to clone the *var*-CHO cDNA sequence. Using the genome sequence data it had been possible to extend the *var*-CHO sequence into the DBL-δ sequence domain. This domain is usually the 3' DBL domain in the *var* gene sequence. Thus it could be predicted that the fragment to be amplified would be a relatively small one. The amplified PCR product was 506 bp (Fig. 5.4). The PCR product was excised from the agarose gel and purified as described in section 5.1.3. The amplified DNA was ligated into a TA cloning vector transformed into competent cells and plated on LB amp plates. 10 colonies were screened by PCR and restriction digest (as above) and positive clones were sequenced.

Sequencing revealed that the PCR band consisted of a number of different sequences. This was expected because the 3' reverse primer (*var* 3) is a degenerate primer. The insert sequence of each clone was aligned against the *var*-CHO clone contig using the Assemblylign™ program. Three of these sequences (each identical to the other) aligned with 100% sequence homology over the 133 bp overlap on the *var*-CHO contig (Fig. 5.5), thus extending the *var*-CHO contig 367 bp into the exon 2 sequence of the gene (Fig. 5.6). The sequence was also submitted to the NCBI Genebank database for blast

analysis. As expected the sequence was found to be homologous to the conserved region of exon 2, providing supporting evidence that the *var*-CHO contig had been extended into exon 2. The *var*-CHO contig was 6619 bp in length. The sequence contig was assembled from these sequence reads on one subsequent occasion to verify the contiguous sequence obtained. (The independent assembly of this sequence by Dr D. Baruch, National Institutes of Health, Washington, and Dr S. Bowman, Sanger Centre, Cambridge has verified the nucleotide sequence of this clone).

Figure 5.4 PCR amplification of cDNA from CHO selected 3D7A using ex2 and var 3 primers

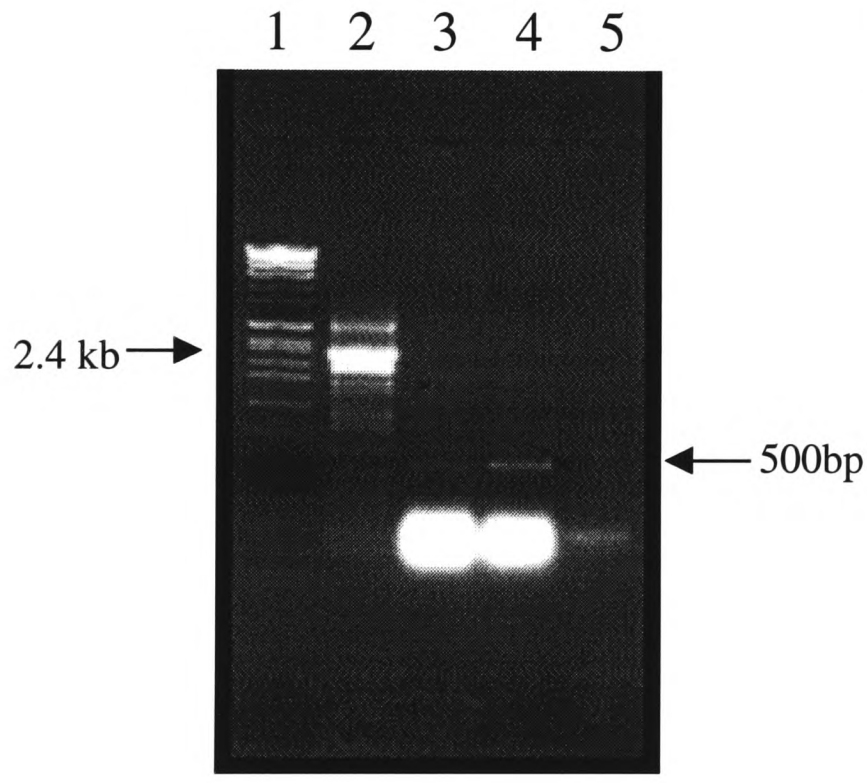

Figure 5.4 PCR amplification of the 3' end of from cDNA, 1. Molecular weight marker VII, 2 . 2.4kb PCR product amplified from DNA (control), 3. Amplification of 500bp PCR product from cDNA, 4. Amplification of 500bp PCR product from cDNA . 5. H_20 (Control).

Figure 5.5 Amino acid sequence of the var-CHO 3' clone

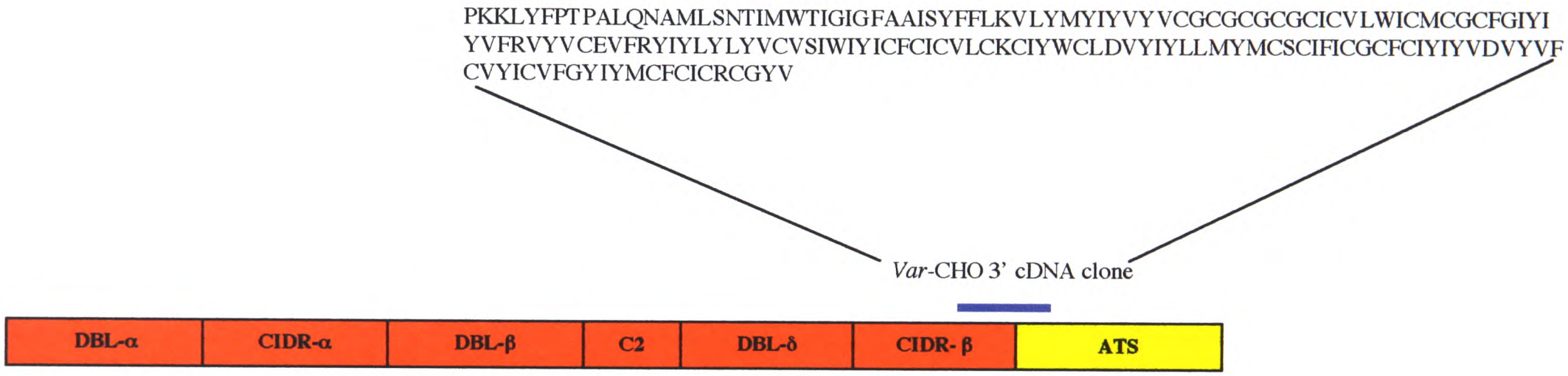

Figure 5.5 Amino acid sequence of the var-CHO 3' cDNA clone superimposed on to a schematic of the exon-one of the var-CHO gene. Blue line indicates position of cDNA clone (not to scale).

Figure 5.6 Full amino acid sequence of the exon 1 of var-CHO

MGPAPRTATTTYSSAKDLLEDIGESVQKEAKKQALGRSESVLHGLLSNATIKGVKNKATKPIQLEYEYHT
NVTGGFDKNNPCANRLDVRFSDIYGGQCTDNKINGNDDETGGTCAPLRRLFLCDQHLSHMKEGNINNTD
NLLLEVSLAAKYEGDSIINNYPDNRDKKEGICTALARSFADIGDIIRGKDLFLVIPKKIEKEKEKVQKNLKRI
FNEIYKKMQDPAKSHYSGDSSDFYKLREDWWALNRKEVWKAITCKAKNDAEYFRKKDSDGKHCSVQN
CKCVDGDPPTNLDYVPQHLRWFDEWSEEFCRKRKDQLKISLEKCRGKNKDKYCSLNGCNCKTTVRGKK
KFDYQQECNDCLVACDPFVHWIDNEKLEFLKQKKKYKNEIKEREPTKKTSHGTINNMYAKEFYETLEKE
HRTVDTFLKLLNEEKECKNHPEVGEGKKTFIDFNDNIETFSHTKICEPCPWCGVKPGGPPWKDNDIDSCG
KKEISFSDKDTTDISILSTDRAKKNILQKLENFCRDTEHINHDIWKCHYENTDNDNCILQNENTGSEKQKIM
PFDAFFFLWLTQMLDDSIEWRKKLKTCINNEKPTNCIRGCKKPCECFERWVEQKEEEWISIEKHFDKQRDI
SEEERYITLEYILNEFFMDKIEKAYGIEKSKELKEKLKSNKGHGIIRDTEHSQDAIKILLEHELEDAKKCTET
HNDEKCKEQEESGGRSLNPDPESDDEEETDNVKENPCAVGKKLTKTVKQIARQMHQAAKKQLGSSSSRA
LKAHAHLGTYKHTNKRDDFKTICKITKDHSNATHNYPQGPCHGKNNDKSMFKTEEGWKPGNQINMNDE
YAFMPPRRQHFCTSNLEYLETADRTLNGIGDDPNVLNHSFLGDVLLAAKFEADFIKEKYNEQSNYKDFSTI
CRAMKRSFADIGDIIKGTDLWDKNGGEQKTQGKLEKIFCKIKNKLPEDIQEKYINDDKNSPQYKKLREDW
WEANRKEVWRAMTCATTSGKIPCSIVTPLDDYIPQRLRWMTEWSEWFCKEQSKLYGELVKDCASCKKK
GKEKCTQGDNDCTPCDKKCKEYGKKIRTWKDQWTKMDGIYQMLYLQAQTTARNAGDTAFDNPNDQY
VIDFFKKLQKANGDNNFGVNTSPYFTPAGYIHQEARVGECEVQKHFCNNNGNQDKYSFRNQPYDHEEAC
ACKKNTKAPEKKKEETPPAGPNVCEIVDKLFEDTTTPHAACQQKYINGHEKFPNWKCVTPSGEKSGDKG
AICVPPRXRRLYIHDLQSLDEKPSDTALRDWFVKSAAVETFFLWHRYKKQKEKKPQEGSLLSGSTLLSQFS
IDLGDEEQPPEKQLAGGKIPDDFLRQMFYTLGDYRDICIGDENVIKTLKYSGDKDDIMEKIQEKIKAVFPTS
GGTSHIPDVKPGGALSRGDWWEQHGKDIWHGMICALTYKETSGSGEKGEKTTITQDGTLKDALLDTDGK
KPKRDYQYTSVTLKEDDSGEKAANVPAKVEPTTLADFTRRPAYFRWLEEWGDGFCRERKKRLAQIKKD
CYEDGGTGEKQYSGYGEACDRTNTSNEGASADLEGPSCANSCSSYRKWIERKKYEFKKQEKAYGGQKQ
NCKKERKAAESNDNDKQFCGTPETTCNTAEAFLQNLGSCKKYNGEGKKIFENTEETFKPAIDCEPCSEFK
VKLEKCNCGSDAKGNTCTTGKITAENFENKTDVNEVVMRVSDNAESGFKGDLKSSCENAHIFEGIKENK
WKCRNVCGYIVCKLEEVNGEKDNGKKILLIRALVTHWVDNFLQDYNKIKKKLNTCMNSSDATPCIKGC
VDKWIKLKKDEWEEIKKPYLEQYKNGYGENYNVKTILEKFQDQPEFKKAIGPCPTLDAFEKSKQCNATAS
SEKGKDGNKSYVIDCLLQELEKLQEKAKKCHDQHSDNPQEKCDDPHPDEPDEEDLLLEEEENTANSAPEI
CKDVIKAPPKQEEKGGCEPASPLEPEEVEEETASVPPGSEPEADKGPVKPAELPKPPKRNKRQPKKLYFPT
PALQNAMLSNTIMWTIGIGFAAISYFFLKVLYMYIYVYVCGCGCGCGCICVLWICMCGCFGIYIYVFRVY
VCEVFRYIYLYLYVCVSIWIYICFCICVLCKCIYWCLDVYIYLLMYMCSCIFICGCFCIYIYVDVYVFCVYI
CVFGYIYMCFCICRCGYV

Figure 5.6 *Var*-CHO exon 1 amino acid sequence. DBL, C2 and CIDR domains are colour coded, DBL-α (303 aa) Blue, CIDR-α (427 aa) Red, DBL-β (278 aa) light green, C2 (134 aa) Pink, DBL-δ (373 aa) Turquoise, (CIDR-β 537 aa), violet, Exon 2 Dark yellow. Cysteine residues are in bold C.

5.2.5 Annotating the exon 1 gene sequence

The complete Exon 1 of the *var*-CHO gene has been sequenced. This sequence encodes for the extracellular portion of the *var* gene. It is this section of the protein that is involved in adhesion. The sequence is 2132 amino acids in length. It contains 102 cysteine residues, which form disulphide bonds and are thus important in maintaining the structural framework of the protein.

The sequence was annotated by hand. The domains which characterise the extracellular domain of the PfEMP-1 protein can be identified by specific sequence motifs. Identification of these sequence motifs within the *var*-CHO sequence permitted the annotation of the sequence. The *var*-CHO sequence contains three DBL domains, two CIDR domains and one C2 domain. The N-terminal domain is a DBL-α domain 303 amino acids in length containing 17 cysteine residues. The DBL-α domain is linked with a CIDR-α domain, 427 amino acids in length containing 17 cysteine residues. These domains have both been implicated in adhesive phenotypes, DBL-α in rosetting via complement receptor-1 (CRI) and heparan sulphate, and CIDR-α in cytoadherence via CD36. The third domain in the sequence is a DBL-β domain, which consistently appears in tandem with the C2 domain. The *var*-CHO DBL-β domain is 278 amino acids in length and contains 13 cysteine residues. The next domain is the C2 domain, 134 amino acids in length and containing 5 cysteine residues. The DBL-β-C2 tandem domain has been implicated in infected erythrocyte adhesion to the endothelial adhesion receptor ICAM-1. The fifth domain DBL-δ is 373 amino acids in length and contains 10 cysteine residues. The final C terminal domain, consistently located immediately 3' of the DBL-δ

domain is the CIDR-β domain, which is 537 amino acids in length containing 40 cysteine residues. The CIDR- β is yet to be associated with an adhesive phenotype, however the high cysteine content may suggest a fundamental role of this domain in maintaining the structure of the protein.

Figure 5.7 3D7A *Var*-CHO gene structure

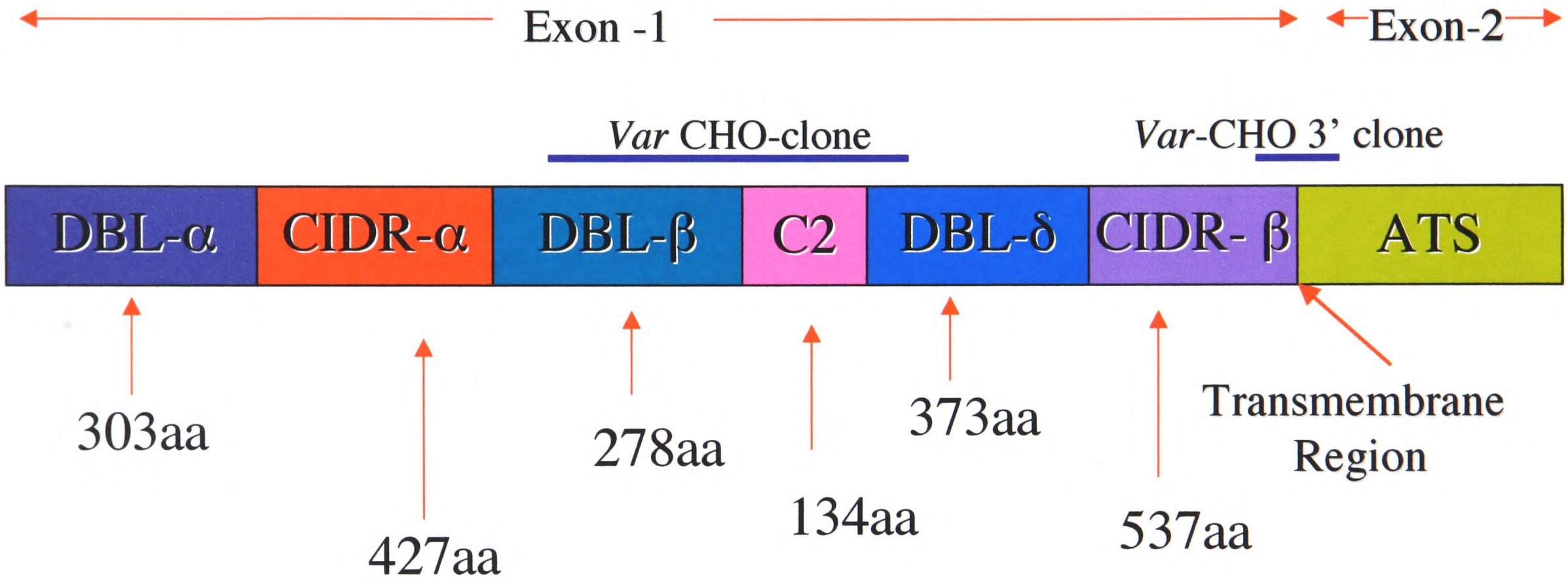

Figure 5.7 Schematic representation of *var*-CHO gene. DBL domains colour coded. DBL-α (303 aa) Blue, CIDR-α (427 aa) Red, DBL-β (278 aa) light green, C2 (134 aa) Pink, DBL-δ (373 aa) Turquoise, (CIDR-β 537 aa), violet. Exon 2 Dark yellow. Positions of *var*-CHO and *var*-CHO3' clones indicated by blue line.

5.2.6 Mapping of the *Var*-CHO gene to Chromosome 13

As detailed above in sections 5.2.3 and 5.2.4 it has been possible to use genomics i.e. the whole genome sequencing project to extend the *var*-CHO sequence. All the genome project derived sequence reads used came from the Sanger Centre malaria genome database. The Sanger Centre is sequencing chromosomes 1, 3, 4, 5, 6, 7, 8, 9 and 13. However, due to DNA contamination of chromosomal DNA from pulse field agarose gel chromosome preparations with DNA from other chromosomes it was not possible to map "in silico" the gene to a specific chromosome. The sequence reads used in the assembly of the sequence came from a number of different chromosome preparations within the Sanger Centre genome database. It was also not always possible to identify the chromosomal origin of sequence reads by the codes used to identify them, a significant problem for "community" access to the genome project databases.

To map the gene to a specific chromosome *var*-CHO clone was radio-labelled and used to probe a pulse field gel separation of 3D7A chromosomal DNA. To avoid any background hybridisation between plasmid DNA and chromosomal DNA the *var*-CHO insert was amplified from their respective plasmids using *var* 5-*var* 8 primers. The PCR products were excised from the gel and purified using methods previously described and resuspended in 50µl H_2O. 2µl of DNA was labelled by random hexamer priming with ^{32}P (See Methods). The labelled probe was then hybridised overnight with a 3D7A pulse field gel blot (provided by Dr D. Arnot). The PFG blot was washed under stringent conditions before being exposed to autoradiographic film.

The *var*-CHO sequence hybridised to a single chromosome of 3D7A on the pulse field blot, chromosome 13. High molecular weight chromosomes 14 and 13 migrate close to each other at the top of the pulse field gel due to their similar molecular weights, separated from chromosome 12 which is significantly smaller (Fig. 5.8,). The Pulse field Gel data also shows that the *var*-CHO sequence is absent from the SD 128 genome. However this sequence or a gene of similar sequence is present on chromosome 13 of the Sudanese clone SD105.

Figure 5.8 Probing pulse field gel of 3D7A, SD 128/5 and SD 105/9 DNA

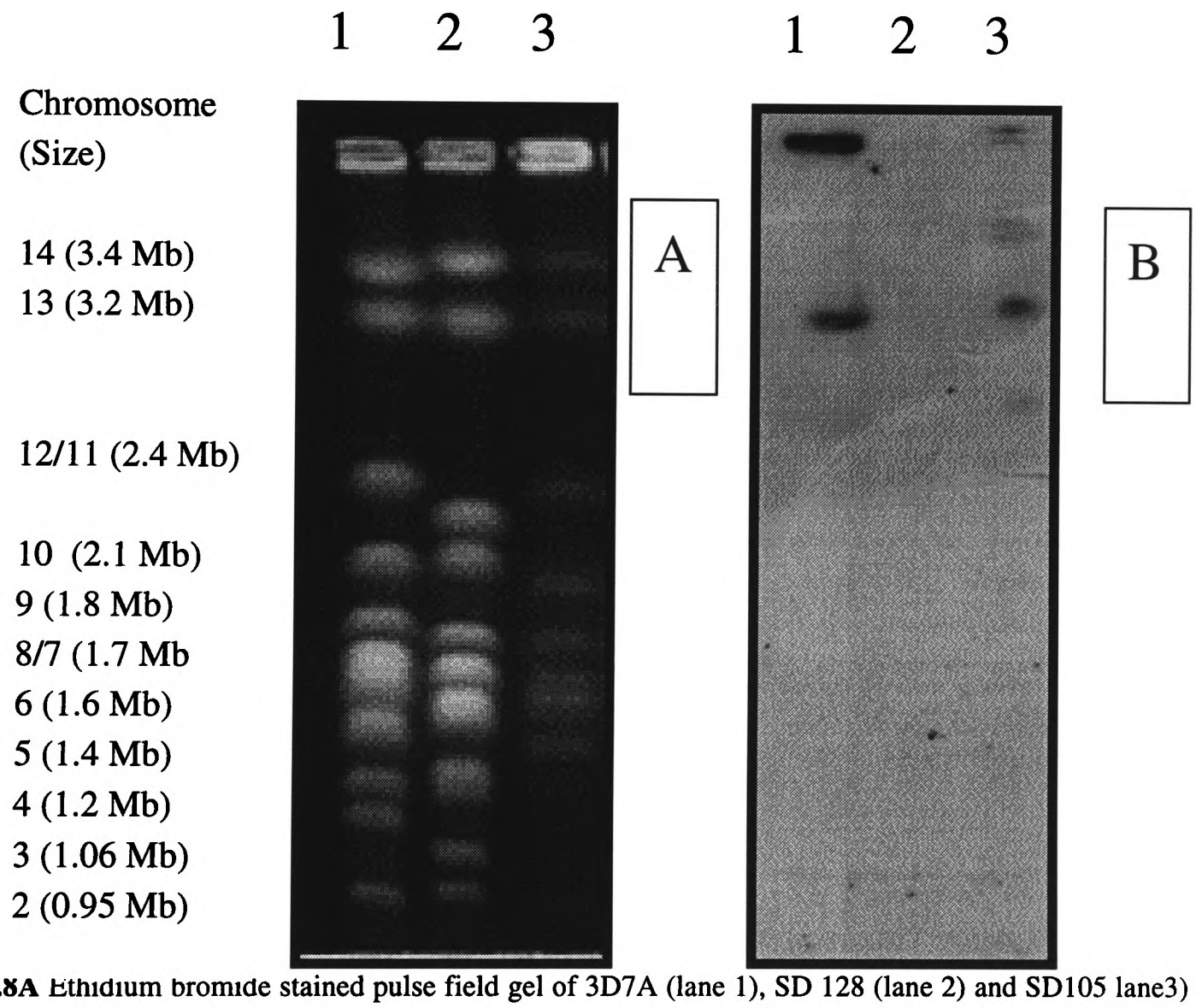

Figure 5.8A Ethidium bromide stained pulse field gel of 3D7A (lane 1), SD 128 (lane 2) and SD105 lane3) DNA. **Figure 5.8B** Southern blot of 3D7A (lane 1), SD 128 (lane 2) and SD105 (lane 3) DNA separated by pulse field gel electrophoresis and transferred to nylon membrane, then hybridised with the *var* CHO cDNA clone.

5.2.7 Verification of sequence alignment

One of the difficulties associated with sequencing multicopy gene families is that there is a potential risk of assembling a contig from a number of homologous but different genes from the same gene family. To verify that the assembled contig represented the 5' and 3' end of the same gene a 3D7A pulse field gel was probed with the *var*-CHO 3' clone. The Var CHO 3' clone also hybridised to Chromosome 13 on the pulse field gel (Figure 5.9) providing supporting evidence that the contiguous alignment of the *var*-CHO sequence represents an alignment of the same gene.

Figure 5.9 Probing pulse field gel of SD 105/9 and 3D7 DNA

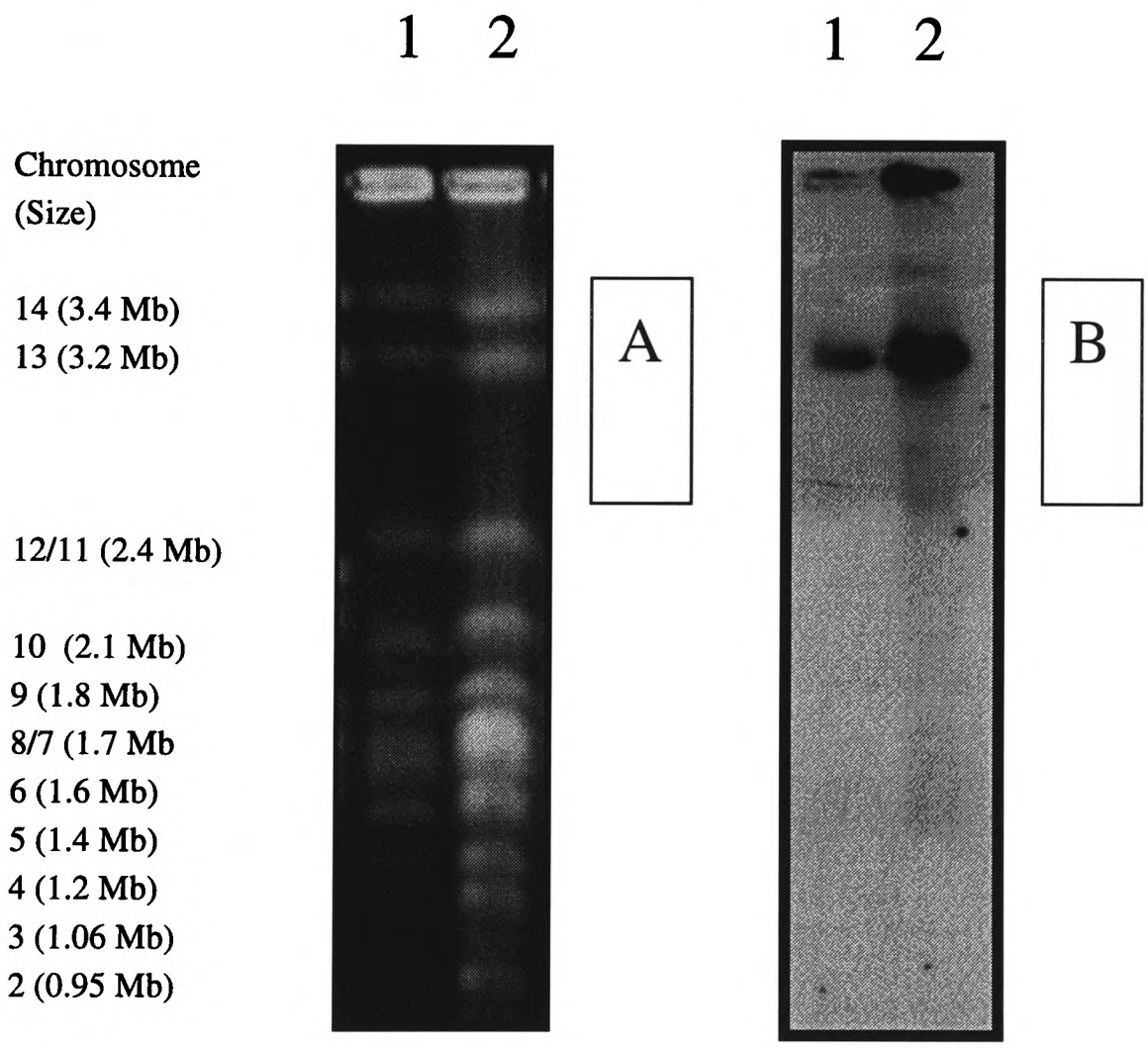

Figure 5.9A Ethidium bromide stained pulse field gel of SD105/9 (lane 1) and 3D7A lane 2) DNA. **Figure 5.9B** Southern blot of SD105/9 (lane 1) and 3D7A lane 2) DNA separated by pulse field gel electrophoresis and transferred to nylon membrane, then hybridised with the *var* CHO 3' cDNA clone.

5.3 Conclusions

PCR amplification of cDNA extracted from parasites selected for increased adhesion to CHO cells amplified a single 1.0kb PCR product. This suggests that selection for a specific adhesive phenotype also selects for the transcription of a specific *var* gene. It was possible to extend the *var*-CHO sequence by accessing the Sanger Centre Malaria Genome Project database using genomics. Most of the exon 1 sequence was obtained from the genome database and the remaining sequence (367 bp) was cloned from cDNA from selected 3D7A. The exon 1 sequence of the *var*-CHO gene is 2132 amino acids long and consists of three DBL domains DBL-α, DBL-β and DBL-δ. This gene represents the first *var* gene to be reported from 3D7A which contains 3 DBL domains. This is also the first *var* gene to be reported from 3D7A with sequence encoding for a DBL-β domain. DBL-γ has been implicated in adhesion to CSA in two independent reports (Reeder *et al*, 1999 Buffet *et al*, 1999). The absence of the DBL-γ domain from the *var*-CHO gene sequence supports the results presented in Chapter 3, that CSA is not involved in this adhesive phenotype. Southern hybridisation of the *var*-CHO clones to a pulse field blot of 3D7A DNA permitted the mapping of the *var*-CHO gene. By using a combination of the pulse field blot data and genomics data from the Malaria Genome Project the *var* gene was given a location of chromosome 13.

Chapter 6
RT-PCR/ dot blot analysis of *var* gene switching in CHO cell selected 3D7A

6.1 Introduction

During the development of this thesis it has been demonstrated that 3D7A can be selected for increased adhesion to the surface of CHO cells, an adhesive phenotype mediated by an as yet unidentified CHO cell surface receptor (Chapter 3). In chapter 4 it was demonstrated that unselected 3D7A cultures transcribe at least 27 different *var* genes. It has also been demonstrated that selection for increased adhesion to the CHO cell surface also selects for the transcription of a specific PfEMP-1gene, *var*- CHO (Chapter 5).

It was predicted that parasites within the CHO cell-selected 3D7A culture would switch the *var* gene being expressed, and thus undergo antigenic variation, during maintenance of the culture in the absence of selection for adhesion. The cytoadhesion assay developed and the 28 sequences cloned during this thesis were therefore employed in a novel assay to try to analyse *var* gene switching in this model system. This involved a RT-PCR/dot blot assay to detect switching from the transcription of the selected *var* gene (*var*-CHO), to the transcription of any of the *var* genes previously cloned from unselected 3D7A. *Var* genes in the parasite clone A4 have been measured to switch at a rate of around 2% per generation (Roberts *et al.,* 1992). These experiments were intended to analyse the rate of *var* gene switching over time in the parasite clone 3D7A

and to test whether there is any repeated pattern in *var* gene switching and thus *P. falciparum* antigenic variation, as has been reported in the primate malaria *P. fragile* (Handunnetti *et al., 1987).*

6. 2 Results

6.2.1 Development of dot blot assay for the hybridisation of *var* transcripts

The dot blot was prepared by binding aliquots of DNA containing equal concentrations of twenty eight cloned *var* gene sequences to a nylon membrane. Twenty seven of these were cloned from an unselected 3D7A parasite culture and the other transcribed *var* gene, *var*-CHO was cloned from 3D7A cultures, selected for increased adhesion to CHO cells. Cloned plasmid DNA from the 28 *var* sequences cloned, was spotted on to a nylon membrane using a dot blot apparatus. DNA on the membrane was subsequently alkali denatured, neutralised and air dried. The DNA was then cross-linked to the nylon membrane by ultraviolet light. To exclude background hybridisation between the plasmid DNA spotted on to the membrane and plasmid DNA contaminating the "probe" during radio-labelling, the cloned *var* gene DNA used for probing the membrane was PCR amplified from the plasmid vector using *var* 5 and *var* 8 primers. The amplified DNA was excised from the agarose gel, purified and re-suspended in 50µl H_20. The pure "probe" DNA was then ready for radio-labelling. 1 µl of *var* gene DNA (approximately 50ng) was labelled with ^{32}P by incorporation of ^{32}P labelled dATP by polymerisation following random hexamer priming. ^{32}P labelled DNA was hybridised to the *var* gene

dot blot membrane overnight. The membrane was then washed under stringent conditions. Briefly following hybridisation, the membranes were washed twice with 2X SSPE, 0.1% SDS for 15 minutes at 65°C, 1X SSPE, 0.1% SDS for 15 mins at 65°C and 0.1X SSPE, 0.1% SDS for 15 minutes. The membranes were then exposed to auto-radiographic film. After trial hybridisation experiments it was decided that 10ng of target DNA produced the cleanest hybridisation signal.

6.2.2 Cross hybridisation of *var* gene clones

To assess the degree of cross hybridisation between *var* gene sequences prior to probing the dot blot with radio-labelled RT-PCR products from the selected 3D7A parasite line, the dot blot was first probed with each cloned *var* gene sequence individually. Using the protocol described above each dot blot was probed in duplicate (Fig. 6.1) .As expected all of the sequences hybridised to themselves, although some cross hybridisation was observed between sequences (Fig. 6.1 & Fig. 6.2). Eight of the 28 DBL-1 derived sequences used to probe the filter cross-hybridised with one other sequence on the filter (Fig. 6.2). Interestingly, none of these sequences cross-hybridised with more than one other sequence, again emphasising the high level of sequence divergence between different members of the PfEMP-1/*var* gene family.

Figure 6.2 Cross hybridisation of var sequences used to probe the var gene dot blot membrane

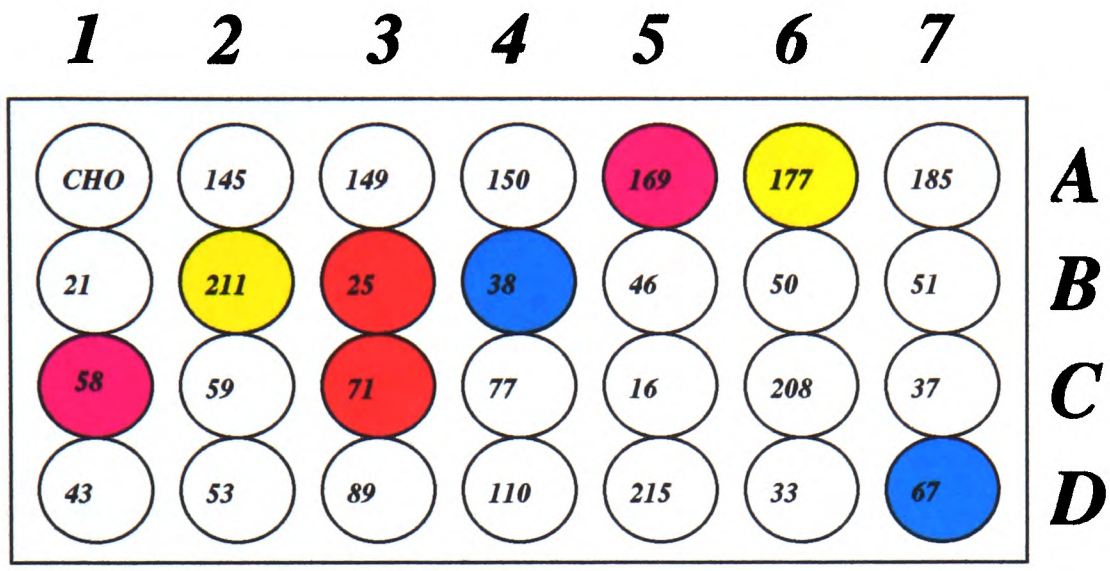

Figure 6.2 Schematic of *var* gene dot blot membrane with cross-hybridising clones indicated. *Var* clones 211 and 177 (Yellow), 25 and 71 (Red), 58 and 169 (Pink) 38 and 67 (Turquoise) cross hybridise with each other. No cross hybridisation was observed between other clones.

6.2.3 RT- PCR amplification of *var* gene transcripts and hybridisation of amplified transcripts to the *var* gene dot blot membrane

Six to eight culture flasks of 3D7A parasites selected for increased adherence to CHO cells were prepared as described in chapter 5. Parasites were grown through 4 generations (96 hours) after selection, before RNA was extracted at ring stage. An aliquot of this RNA was used to synthesise cDNA using the *var* 3 primer. A fraction of the cDNA was used in a PCR reaction. The band produced by this PCR reaction was 1.0

Figure 6.1 A. Duplicate dot blot of var gene sequences probed with var 177 sequence, which cross hybridises with var 211 sequence.

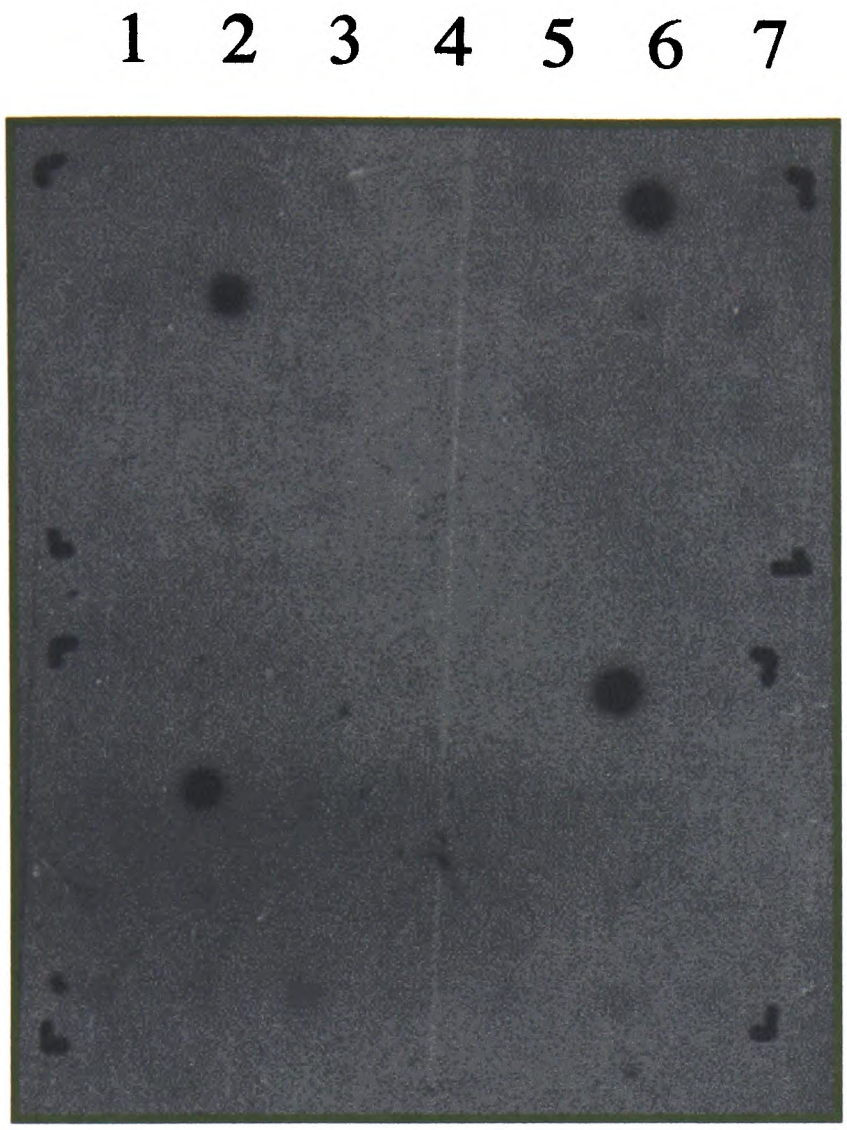

Figure 6.1 Duplicate *var* gene dot blot membrane probed with ^{32}P radio-labelled *var* 177 sequence. *Var* 177 sequence hybridises to *var* 177 clone spotted on to membrane (membrane 1: 6A, membrane 2: 6F). The *var* 177 clone cross hybridises with *var* 211 (membrane 1:2B, membrane 2: 2G).

Figure 6.1 B. Schematic of var gene dot blot filter

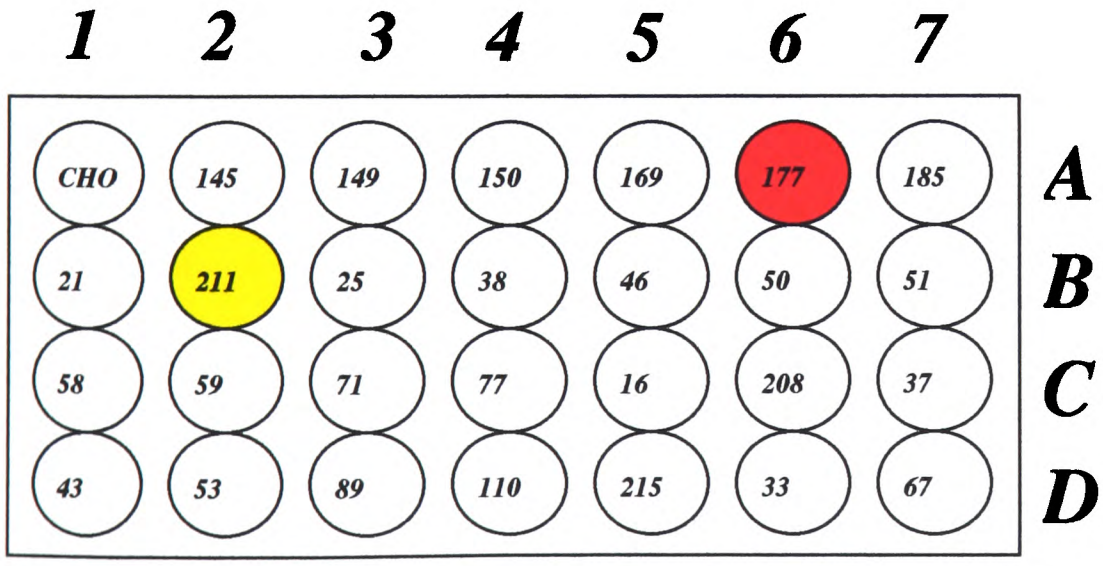

Figure 6.1 B Schematic of *var* gene dot blot membrane with positions of transcribed *var* sequences. Position that *var* 177 hybridises to (Red) and the position the *var* 177 sequence cross-hybridises to (Yellow) are indicated.

kb, smaller than the original 27 clones, cloned in chapter 4, but consistent with the size of the PCR band amplified after selection for adhesion to CHO cells (Fig 6.3) and (chapter 5).

Figure 6.3 RT- PCR amplification of RNA extracted from selected 3D7A parasites 4 generations after selection

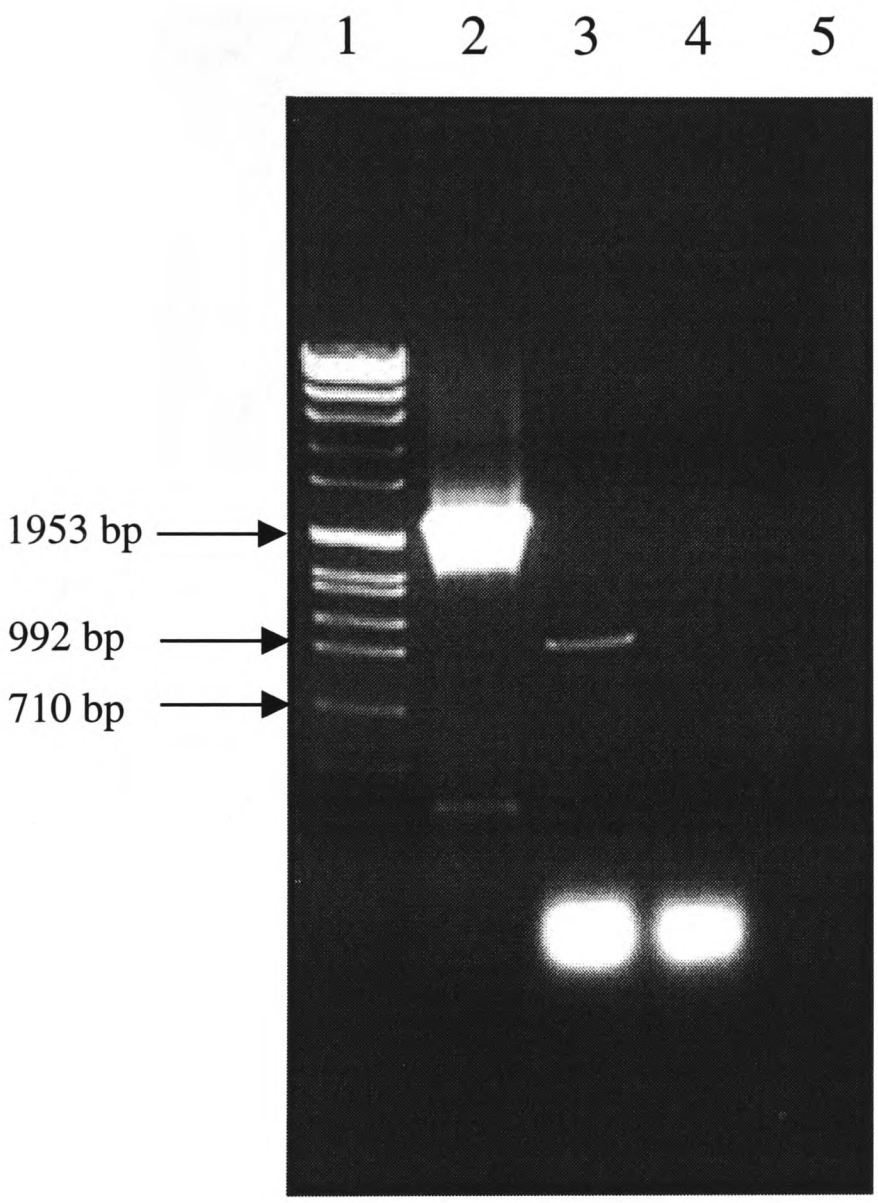

Figure 6.3 RT-PCR of 3D7A parasites selected for adhesion to CHO cells, 1. molecular weight marker VII, 2. 2.4 kb PCR product amplified from DNA, 3. 1.0 kb PCR product amplified from cDNA reverse transcribed from RNA extracted from CHO cell, selected 3D7A, 4 generations after selection, 4. RNA (not reverse transcribed).5. H_20.

Figure 6.4 A. Dot blot hybridisation of RT-PCR product amplified from RNA extracted from 3D7A selected parasite line, 4 generations after selection.

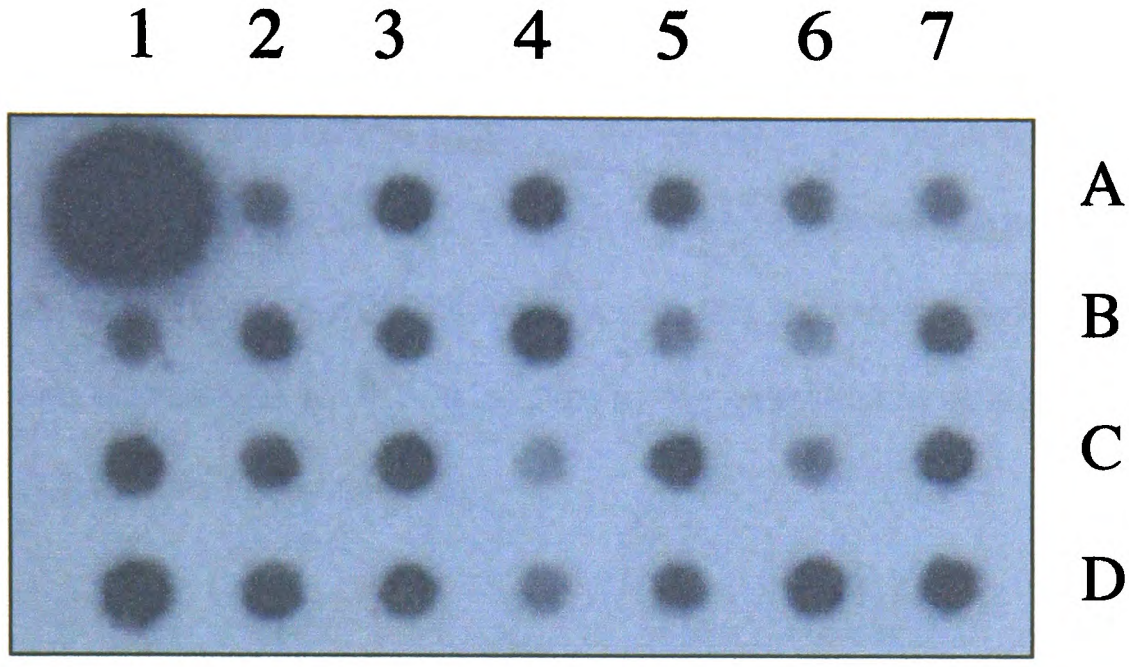

Figure 6.4 B

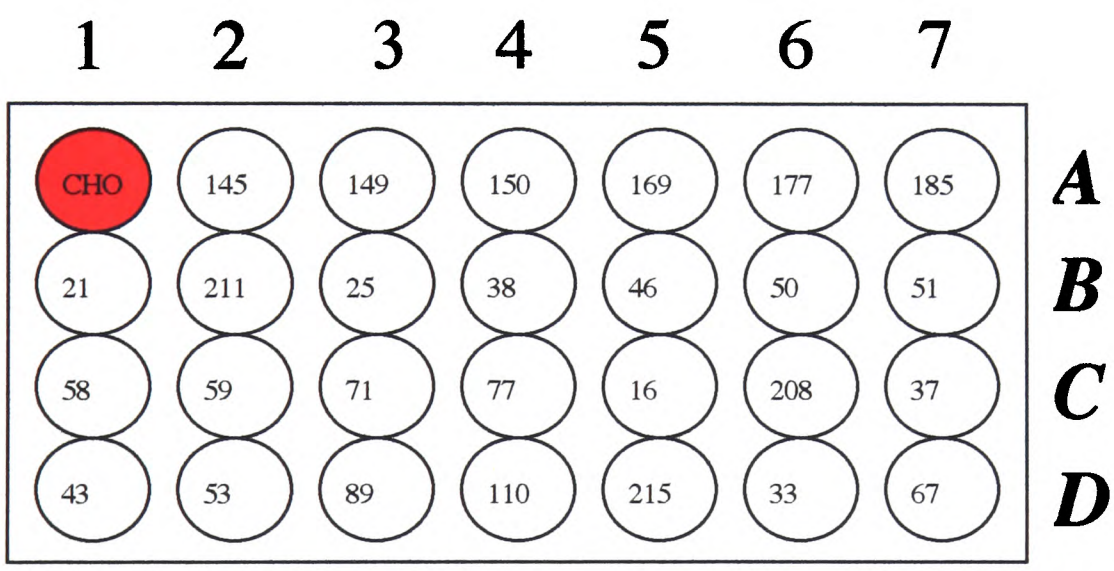

Figure 6.4 A. RT PCR of 3D7A, 96 hours (4 generations) after selection for adhesion to CHO cells .RT-PCR product hybridises to *var* gene dot blot membrane at the position of the *var*-CHO sequence, top left hand corner (1A). **Figure 6.4 B.** Schematic representation of dot blot with position of *var* sequences shown, hybridisation signal of RT-PCR product shown in red.

The remaining PCR product was labelled as described in 6.2.1 and hybridised to the dot blot membrane. The amplified sequence hybridised only to the *var*-CHO sequence on the *var* gene dot blot membrane (Fig. 6.4) providing further evidence that the predominant transcribed sequence amplified was that of the *var*-CHO gene. Using the proposed switch rate of 2% per generation (Roberts *et al* 1992) and assuming equal growth rates for all variants, after 4 generations it was predicted that about 8% of the parasite population could have switched to the expression of another *var* gene variant. At such an early stage after selection it may not be possible to detect a switch in the in *var* gene expression by RT-PCR, due to the exponential amplification of abundantly transcribed *var*-CHO sequence.

Figure 6.5 RT- PCR amplification of RNA extracted from selected 3D7A parasites 8 generations after selection

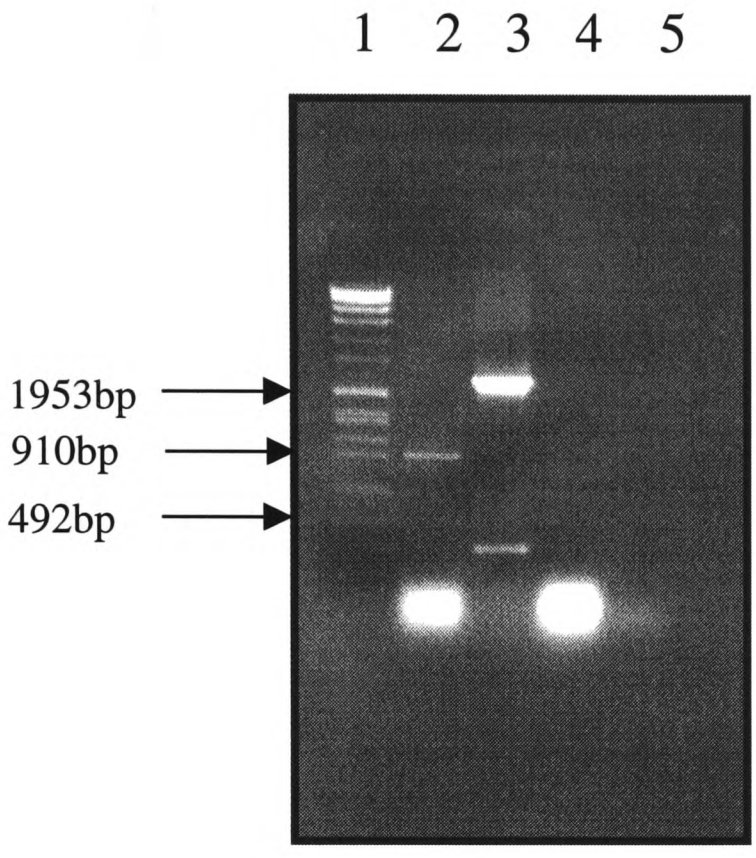

Figure 6.5 RT PCR of RNA from 3D7A selected for adhesion to CHO cells 8 cycles after selection , 1. molecular weight marker VII, 2. 1.0 kb PCR product amplified from cDNA reverse transcribed from RNA extracted from CHO cell binding 3D7A parasites 8 generations after selection, 3. 2.4 kb PCR product amplified from DNA (control), 4. RNA (not reverse transcribed), 5. H_20 control.

Figure 6.6 A. Dot blot of PCR amplified cDNA from selected 3D7A, 8 generations after selection for adhesion to CHO cells

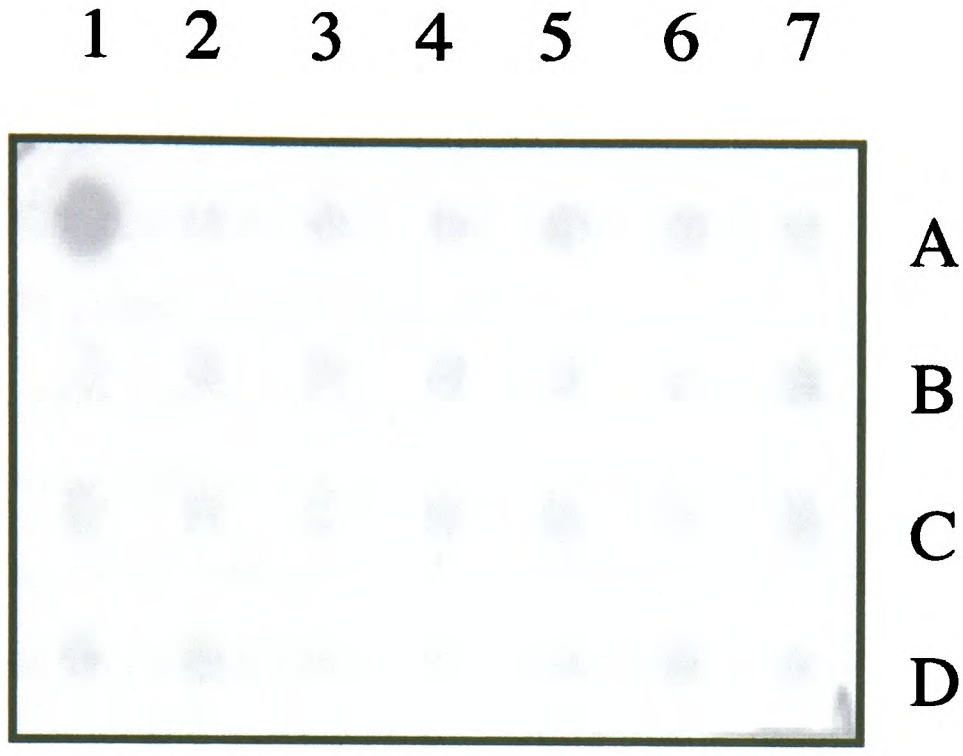

Figure 6.6 B

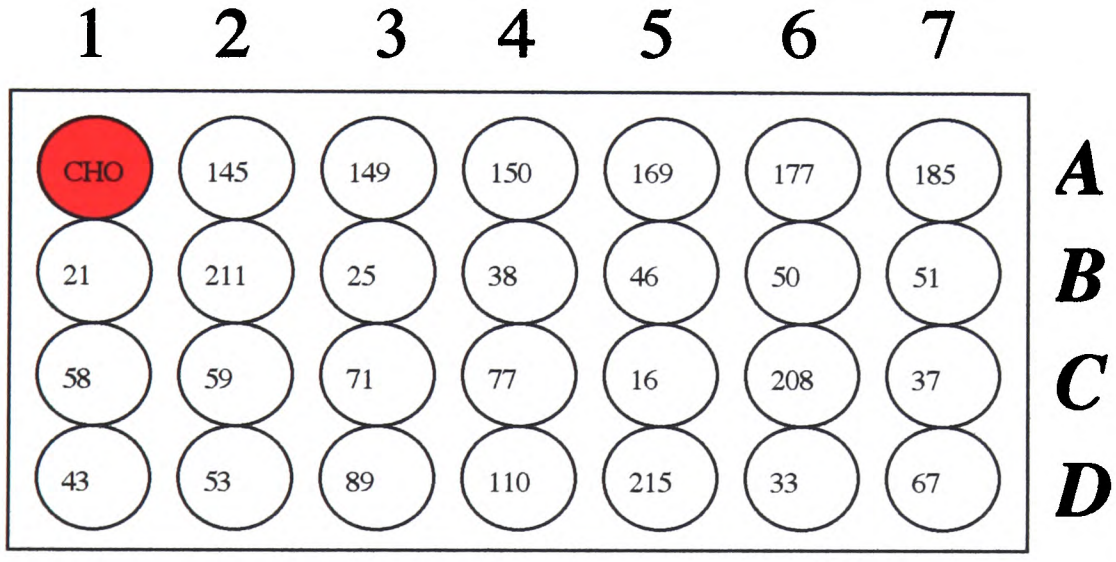

Figure 6.6 A. RT-PCR of 3D7A, 8 generations after selection for CHO cell adhesion. The RT-PCR product hybridises to CHO sequence on dot blot membrane ,Top left hand corner,(1A).
Figure 6.6 B. Schematic of *var* gene dot blot with positions of *var* sequences indicated. Position of RT-PCR hybridisation shown in red.

To continue monitoring *var* gene switching over an extended period of time, an aliquot from this parasite culture was grown for a further 4 generations, before the ring stage RNA was again extracted and RT-PCR amplification performed (Fig. 6.5). Again a small PCR product, 1.0 kb was amplified, consistent with the transcription of *var*-CHO. No PCR band was detected at 2.4 kb. This suggests that 8 generations after selection it was still not possible to detect any change in the transcription of *var* genes in 3D7A. The RT-PCR product to total extracted RNA was again labelled with ^{32}P and used to probe the *var* gene membrane. The only sequence to be highlighted by the product of this PCR was the *var*-CHO sequence providing further evidence that the predominant expressed sequence being detected by this experiment was the *var*-CHO sequence (Fig. 6.6). To continue the analysis an aliquot of parasites were cultured for a further 4 generations and again total RNA was extracted, used in an RT-PCR reaction and the products used to probe the *var* gene membrane. No change in the pattern of RT-PCR or dot blot hybridisation was observed. 7 further RNA extractions, RT-PCR amplifications and hybridisations were performed every fourth generation until the parasites had gone through 40 generations in total. After 40 generations, based on a switch rate of 2% per generation it is predicted that 55% of the parasite population would have switched to the expression of another *var* gene. After 40 generations the PCR product (Fig. 6.7) and the dot blot (Fig. 6.8) remained unchanged in their pattern of expression It was thus not possible to detect any change in *var* gene transcription once the CHO cell adhesion phenotype had been established. The experiment was subsequently terminated and the parasites preserved in liquid nitrogen.

Figure 6.7 RT- PCR amplification of RNA extracted from selected 3D7A parasites 40 generations after selection

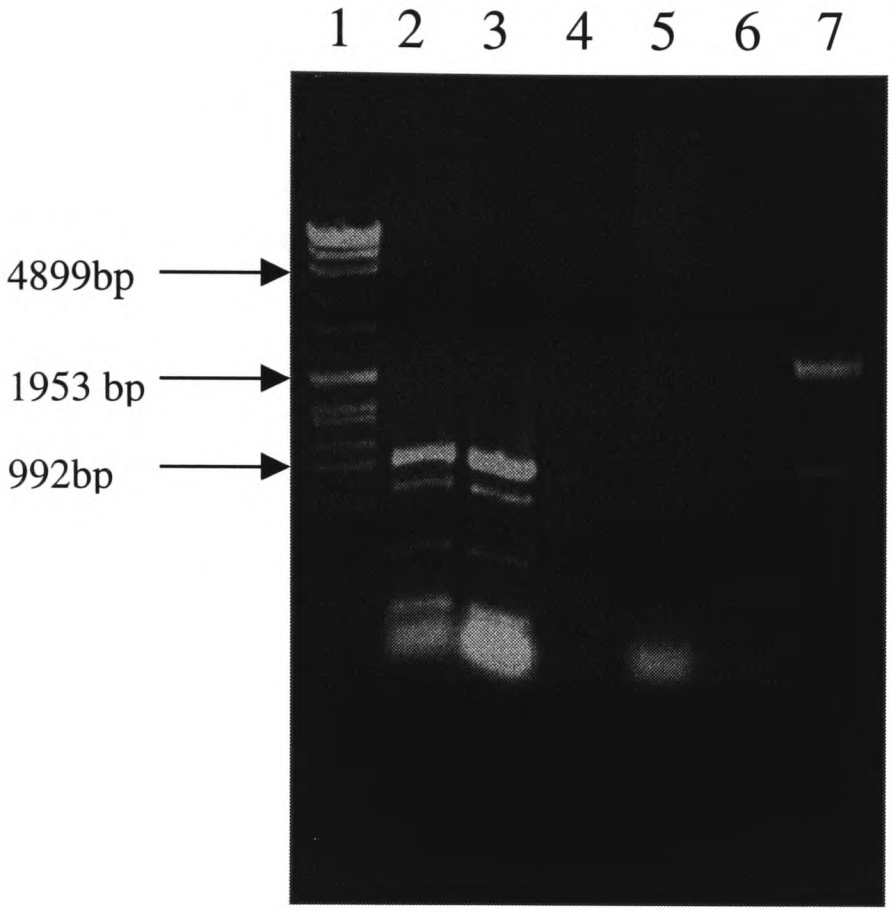

Figure 6.7 RT PCR of 3D7A parasites selected for adhesion to CHO.,1 molecular weight marker VII, 2. 1.0 kb PCR product amplified from cDNA extracted from parasites 36 generations after selection for adhesion to CHO cells, 3. 1.0 kb PCR product amplified from cDNA extracted from parasites 40 generations after selection for adhesion to CHO cells, 4. RNA (not reverse transcribed), 5. RNA not reverse transcribed), 6. H_20 and 7. 2.4 kb PCR product amplified from DNA (control).

Figure 6.8 A. Dot blot of RT-PCR products amplified from 3D7A parasites 40 generations after selection for adhesion to CHO cells.

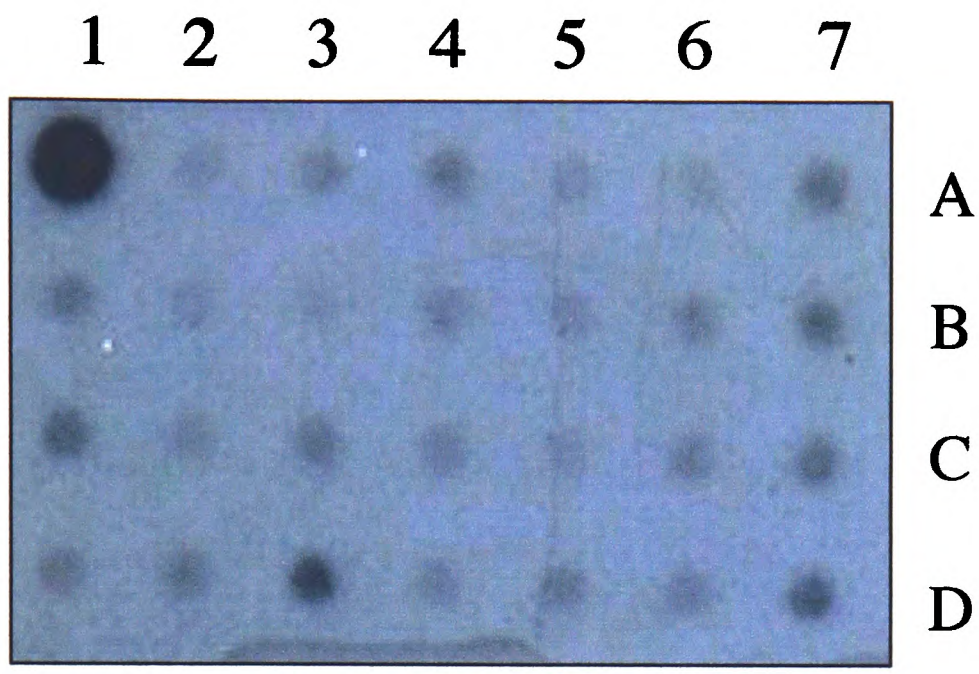

Figure 6.8 B

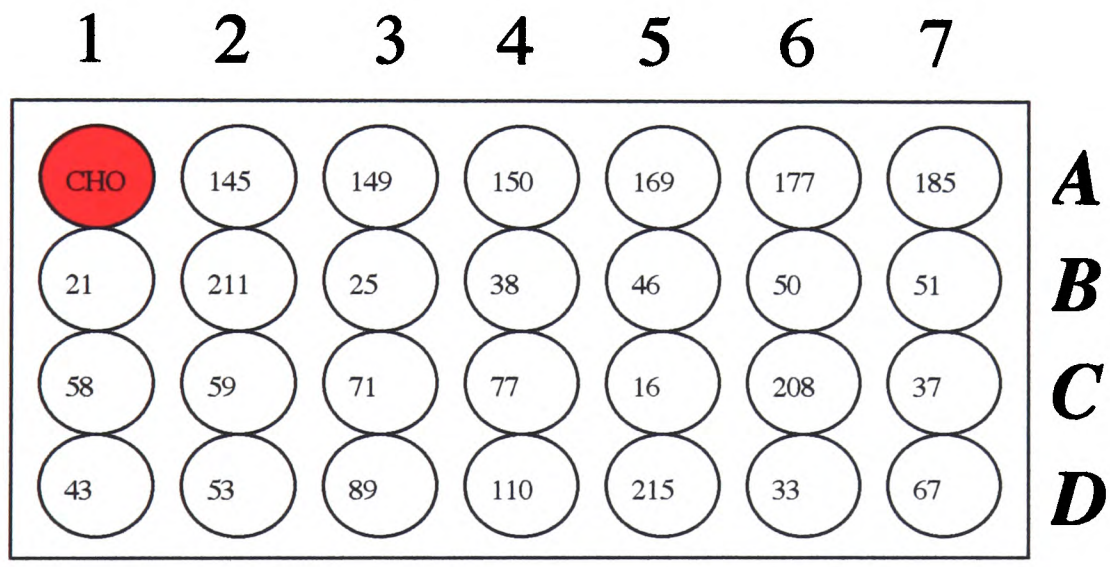

Figure 6.8 A. RT-PCR of 3D7A 40 generations after selection for CHO cell adhesion RT-PCR product hybridises to CHO sequence on dot blot membrane ,Top left hand corner,(1A).
Figure 6.8 B. Schematic of *var* gene dot blot with positions of *var* sequences indicated. Position of RT-PCR hybridisation shown in red.

6.2.4 Loss of cytoadhesion of selected 3D7A to CHO cells over 40 generations

Antigenic variation in *P. falciparum* occurs at approximately 2% per generation, however it was not possible to detect any antigenic variation using the dot blot assay, therefore, another approach was used to detect *var* gene switching. It was predicted that every generation approximately 2% of the CHO cell, selected 3D7A parasite culture would which away from the expression of the *var*-CHO gene mediating adhesion to CHO cells in 3D7A. Over time an accumulation of these switches should result in a reduction in the adhesion of the parasite culture. To test this hypothesis an experiment was conducted to test the adhesive ability of CHO cell selected 3D7A, cultured for several generations in the absence of selection. The binding of selected 3D7A parasites that had been in culture for 40 generations without selection (G40) was compared to the adhesion of selected 3D7A parasites that had been recently selected for cytoadhesion (G0). These two parasite lines were synchronised by pre-treatment with 5% sorbitol (see methods) and cultured to 10% parasitaemia at early trophozoite stage. CHO cells were prepared as described in chapter 5. The G0 and G40 3D7A parasites were incubated with semi-confluent CHO cells for 1 hour with gentle agitation every 15 minutes. Parasites not bound to CHO cells were subsequently washed off and the petri dishes were fixed and stained as previously described. The binding assay was conducted in duplicate and was repeated 3 times. The results of these experiments are displayed in Table 6.2. After forty generations of growth the 3D7A population still bound to the CHO cells to a greater extent than the control unselected 3D7A culture (Table 6.1, $P = 0.0043$). This binding was some what less that the binding of the G0 parasite culture (Fig. 6.9/Table

6.1, T Test P = 0.0014). These experiments thus suggest that a considerable proportion of the selected 3D7A parasites had lost their ability to bind over the 40 generations they had been in culture. Presumably due to the switching in the expression of *var* genes in culture.

Figure 6.9 Bar graph of adhesion of 3D7A parasites 0 generations versus 40 generations after selection

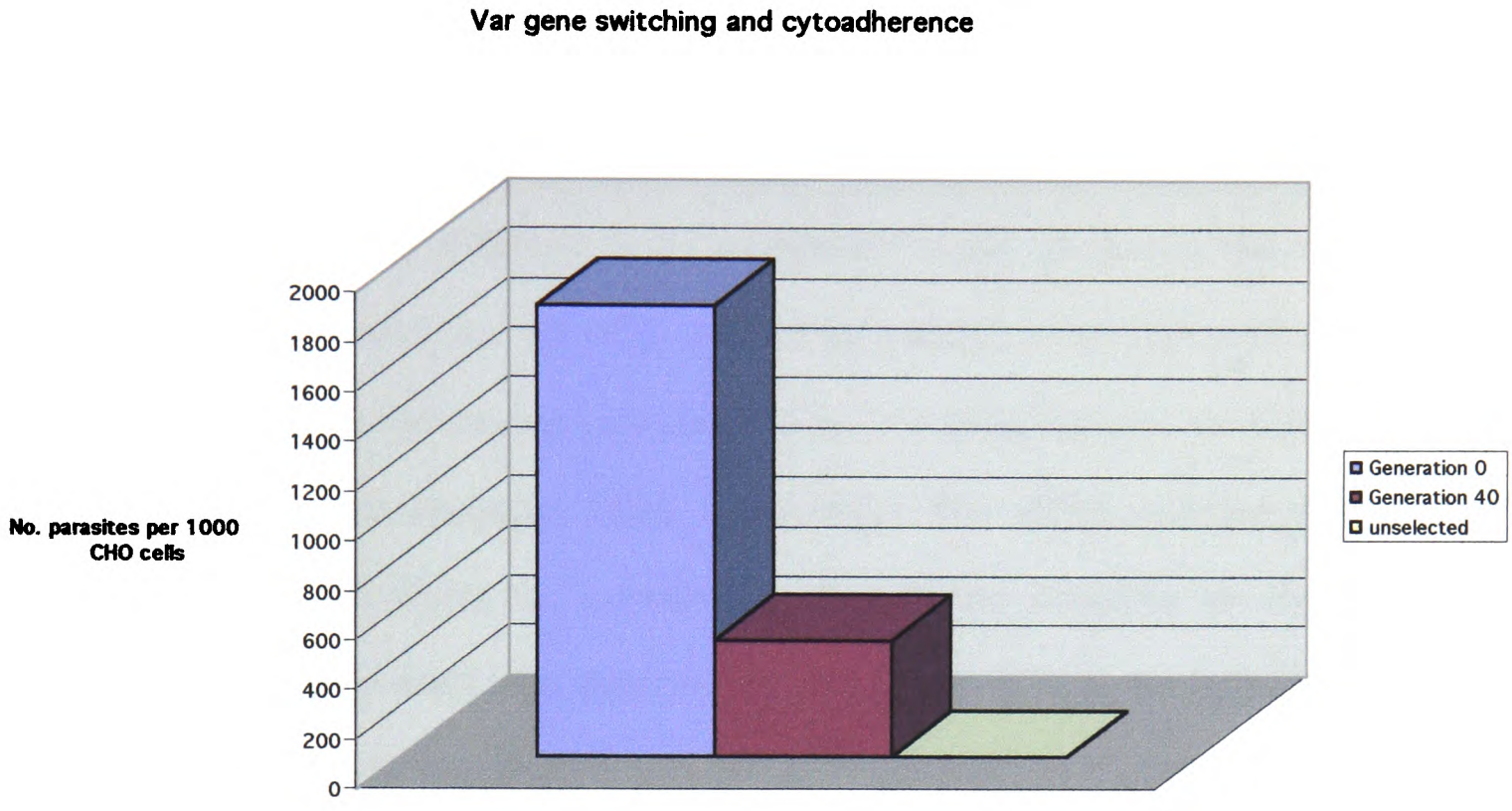

Table 6.1 . Generation 0 and generation 40 parasites and adhering to CHO cells

PARASITES	PARASITES BOUND PER 1000 CHO
Generation 0	1820.25 ± 484.3
Generation 40	471.5 ± 119.3
Unselected 3D7A control	2.5 ± 1

Table 6.1 3D7A parasites selected for adhesion to -CHO cells. Generation 0 (G0) parasites recently selected for adhesion to CHO cells. Generation 40 (G40) parasites selected for adhesion of CHO cells and allowed to grow without selection for 40 generations, experiments were conducted in duplicate N = 6; ± = Standard Deviation.

6.3 Conclusions

Selection of 3D7A for adhesion to the CHO cell surface results in the expression of a specific *var* gene, *var* CHO. RT-PCR amplification of the transcribed RNA resulted in the amplification of a PCR band 1.0 kb in size, consistent with the amplification of the *var*-CHO gene product (Chapter 5). Hybridisation of this product to the *var* gene dot blot membrane revealed that the PCR product hybridised to the *var*-CHO sequence on the *var* gene dot blot membrane, providing further evidence that the *var*-CHO transcript was the predominant sequence being transcribed. The 3D7A selected parasite was cultured without selection for a further 4 generations, however no change in the transcript being expressed was detected either by RT-PCR or by hybridisation. The selected 3D7A parasite remained in culture for 40 generations without selection with no detection of *var* gene switching by the assay developed.

One possible interpretation of these results is that the parasites were not switching the *var* gene being expressed. An another explanation for the failure of the RT-PCR/dot blot assay to detect *var* gene switching could be that the dot blot membrane was not able to detect the *var* genes to which these parasites were switching. The *var* gene dot blot membrane only contains 28 of a possible 50 3D7A *var* genes. It is possible that the parasites had switched to the transcription of one of the 22 possible *var* genes not represented by the dot blot membrane. To account for the inability of this assay to detect 2/5ths of the possible 50 *var* genes, the equation used to calculate the proposed switch rate of 2% per generation was modified.

The equation used to calculate the switch rate in *P. falciparum* antigenic variation was

$(1-r)^n = P$ (Roberts *et al*., 1992)

r = rate of switching (2%)

n = Number of generations

P = Number of parasites expressing the original gene expressed

This equation was modified and the detectable switch rate over n generations was calculated using the equation.

$(1-3/5*r)^n= P)$.

Using this equation the detectable switch rate of this RT-PCR dot blot assay becomes 1.2% per generation. After 40 generations the dot blot assay developed should detect that 38% of the parasite population had switched to the expression one or more of the other 27 *var* genes cloned. No switch was detected, thus a second explanation may be more plausible. The *var*-CHO clone contains a DBL-β sequence, which is always 3' of the DBL-α sequence and thus RT-PCR amplification of the *var*-CHO sequence produces a PCR product half the size of the other 27 clones generated. The region amplified in the *var*-CHO gene (DBL-β -DBL-δ) is shorter and thus more efficient to amplify than the longer DBL-α- DBL-δ region amplified in the 27 other *var* gene sequences cloned. *Var*-CHO transcripts present amongst the 27 DBL-α - DBL-δ transcripts will be therefore preferentially amplified. As the parasites remain in culture for a number of generations without selection they may have switched the *var* gene being expressed, however the system developed may not have detected any switch because the *var*-CHO sequence was amplified in preference to the other 27 sequences,

even when it represented a minority of total cDNA transcripts present. A better experimental design would possibly be to directly label cDNA and use this to hybridise the *var* gene dot blot membrane. This may avoid the possible problems involved in preferential PCR amplification of specific transcripts.

The analysis of the cytoadherence characteristics of the freshly selected generation zero parasites and the generation 40 parasites, which had been in culture for 40 generations without selection differed. Over a period of time the number of parasites binding per 1000 CHO cells was reduced in the generation 40 parasites when compared to the generation zero parasites. This observation is consistent with the hypothesis that some of the parasite population have switched away from the expression of *var* CHO to the expression of another *var* gene, one not involved in this adhesive phenotype.

Chapter 7
Discussion

The cloning of the *var* gene family in 1995 was arguably the most significant achievement in the recent history of malaria research, not only due to technical difficulties involved in cloning these genes, but also because of the biological importance of the proteins the genes encode. The weight of current evidence supports the view that PfEMP-1 proteins represent an important immune evasion strategy evolved by the parasite to avoid elimination by the host. If one defines antigenic variation to be the capacity of a single cell or organism to spontaneously change it's antigenic composition without any accompanying change at the genetic level, then these proteins mediate antigenic variation (Roberts *et al.,* 1992). This may be the main mechanism used by *P. falciparum* to maintain chronic infection within the host, thus facilitating transmission to the mosquito vector.

The immune response against the infected erythrocyte surface which is currently assumed to be largely against PfEMP-1 protects against clinical malaria, thus understanding the immune response against these proteins is important (Marsh *et al.,* 1989; Bull *et al.,* 1998 Giha *et al.,* 2000). PfEMP-1 also mediates infected erythrocyte adherence to uninfected erythrocytes (rosetting) and infected erythrocytes (auto-agglutination). The selective advantage that these adhesive phenotypes confer on to the parasite is not known, but both adhesive phenotypes have been associated with severe disease, (Carlson *et al.,* 1990, Roberts *et al.,* 2000). Disregulation of normal dendritic cell function by adhesion of infected erythrocytes to the dendritic cell surface is also thought to be a PfEMP-1 mediated

phenotype (Urban *et al.,* 2000), a phenotype which may also be important in the pathogenesis of severe disease. In addition PfEMP-1 mediates endothelial cytoadherence, which may result in the sequestration of mature infected erythrocytes in the post-capillary vascular beds of vital organs such as the brain, precipitating cerebral complications, and the placenta, increasing susceptibility to malaria infection during pregnancy.

Thus the study of PfEMP-1 and the regulation of expression of the gene family that encodes these proteins is necessary to increase understanding of the basic biology of the parasite, which it is hoped, will facilitate the development of vaccines and anti-adhesive therapies. The aim of this study has been to study PfEMP-1mediated cytoadherence and antigenic variation in *P. falciparum.* What follows is a discussion of the results obtained during these investigations.

7.1 Cytoadherence in 3D7A

In 1995 Rogerson and his colleagues developed an adhesion assay for the investigation of chondroitin sulphate A-mediated adhesion of infected erythrocytes to CHO cells (Rogerson *et al.,*1995). It was possible to use their assay to select 3D7A parasites capable of binding to the surface of CHO cells. Unselected 3D7A binds to CHO cells at very low levels, but this can be substantially increased by several rounds of selection.

To assay for the specificity of binding of selected 3D7A to CHO cells, free CSA in solution was used to inhibit cytoadhesion, essentially the experiment which was originally used to define CSA as a cytoadherence receptor (Rogerson *et al.,* 1995). CSA in solution

failed to inhibit binding of 3D7A infected erythrocytes to the surface of CHO cells. This result was supported by experiments employing the commercially available enzymic mixture, chondroitinase ABC to hydrolyse CSA from the CHO cell surface This also failed to inhibit binding. A third assay using a CHO cell line defective in glycolysation (CHO-745) was conducted. CHO-745 dose not express GAGs due to a deficiency in the production of xylosyltansferase (Esko *et al* .,1985). These cells cannot support chondroitin sulphate A mediated adhesion. The 3D7A parasites selected for normal CHO cell binding were able to bind to these mutant cells. These results constitute evidence that 3D7A infected erythrocytes adhere to CHO cells in a chondroitin sulphate A independent manner. This is consistent with the observation from the Gysin laboratory that 3D7A does not adhere to CSA expressed at the surface of *Saimiri* brain endothelial cells (SEBC) (Pouvelle *et al* 1998). The data presented in this thesis suggest that that there is another cytoadhesion receptor, in addition to CSA expressed at the surface of CHO cells. The question is what is this cytoadherence receptor?

To answer this question the combination of enzymatic digestion of CHO cells, inhibition of adhesion by free carbohydrate in solution and adhesion to the CHO-745 mutant cell line were also used to investigate the possibility that other GAGs expressed at the surface of CHO cells were mediating the observed adhesive phenotype. GAGs investigated include another chondroitin sulphate of similar structure, chondroitin sulphate C, the rosetting receptor heparan sulphate, and the placental adhesive receptor hyaluronic acid. None of these potential candidates were found to mediate the observed adhesive phenotype.

Sialic acid was also investigated as a candidate cytoadherence receptor. Sialic acid has not

been identified as a cytoadherence receptor, however it is used by the merozoite stage of the parasite during erythrocyte invasion (Adams *et al.,* 1992). It is also used by the protozoan parasite *Typanosoma cruzi* to invade mammalian cells (Ming *et al.,* 1993). Enteropathogenic *E. coli* and Adenovirus type 37 also use this carbohydrate as an adhesion receptor (Vanmaele *et al.,* 1994; Arnberg *et al .,* 2000). Binding inhibition experiments conducted excluded sialic acid as the cytoadherence receptor mediating the observed cytoadherence phenotype.

In addition to these carbohydrates there are a number of proteins expressed at the endothelial surface which have been identified as host receptors mediating cytoadherence. They represent possible candidates for the adhesive receptor mediating CHO cell adhesion in this model. The leukocyte differentiation antigen CD36 has been demonstrated to be quantitatively the most important infected erythrocyte cytoadherence receptor (Newbold *et al.,* 1997). Many members of the immunoglobulin superfamily, involved in the transmigration of leukocytes, are also infected erythrocyte cytoadherance receptors. ICAM-1, VCAM-1 and ECAM-1 have been identified as infected erythrocyte cytoadherence receptors (Berendt *et al.,* 1989; Ockenhouse *et al.,* 1991). Other endothelial proteins identified as cytoadherence receptors include thrombospondin, PECAM-1, complement receptor-1 and P-selectin. (Roberts *et al.,* 1985; Treutiger *et al.,* 1997; Rowe *et al.,* 1997; Ho *et al.,* 1998). None of these cytoadherence receptors however are known to be expressed at the surface of CHO cells (Makrides *et al.,* 200; Hasler *et al.,* 1993; Johnston *et al.,* 2000; Cruthfield *et al.,* 2000; Raingner *et al.,* 2000;Pouvelle *et al.,* 2000) and are unlikely candidate receptors for this adhesive phenotype. The authors referred to above attempted to detect the presence of these cytoadherence receptors at the surface of

CHO cells using antibody to human endothelial proteins. It can not be excluded that CHO cells constitutively express hamster homologs of these human endothelial receptors which are not recognised by antibodies to human endothelial receptors. Thus to completely exclude these receptors as possible candidates, it may be necessary to test cytoadhesion to each of these receptors individually either by adhesion to these receptors attached to plastic petri-dishes or recombinant protein expression at the surface of cell lines such as CHO or cos-7 cells. Given more time these experiments would constitute part of a plan of further investigation. The experiments detailed however strongly suggest that cytoadhesion to CHO cells in 3D7A in mediated by a undefined cytoadherence receptor, a finding which has great implications for scientists in this field using CHO cells as an adhesion model for CSA binding (Sherf *et al* .,1995). Selection for adhesion to this cell type may be selecting not for one, but multiple adhesive phenotypes. Thus the use of a reliable purified protein based adhesion assay to validate the such obervations based on cellular assays is essential.

7.2 *Var* genes transcribed by 3D7A

Using RT-PCR it was possible to amplify at least 27 *var* genes from ring stage unselected 3D7A. These sequences all contain 2 DBL domains, DBL-α and DBL-δ. Given that the expression of PfEMP1 at the erythrocytes surface is perceived to be an immune evasion strategy employed by the parasite why are so many *var* genes being expressed within a single culture? Is this a true reflection of the *var* gene expression in vivo?

It is possible that in vivo antigenic variation occurs as it has been described in vitro (Roberts *et al* ., 1992), However in vivo the rate of switching or rather the exposure of a

particular antigenic type to immune system may be modulated by the hosts immune system. Within an antigenically mixed population only parasites to which the host is immunologically naïve can proliferate. These parasites proliferate whilst the host's immune response against its antigenic determinants is developing. When the immune responses against the PfEMP1 type being expressed is fully developed these parasites are destroyed by the host immune response. Parasites that have switched their *var* gene expression escape this immune response. If the switching process is random, parasites which switch to an antigenic determinant which is recognised by the host are immediately destroyed. However those who switch to a novel antigenic determinant (with respect to the hosts immunity) survive, perpetuating another "cycle" of infection. This may explain the oscillating infection observed in a number of animal experiments (Brown & Brown 1965, Hommel *et al.,* 1983 Barnwell *et al.,*1983), whilst in keeping with the observation of a large number of *var* gene transcripts being expressed in vitro. Another possible explanation for this "random" expression of a number of *var* genes is that *var* gene switching is not random. The mosquito stage in the parasites life cycle may"reset" the *var* gene expression cycle such that on entry in to the vertebrate host (or culture) the *var* gene expression is set to the expression of a single *var* gene. However over a period of time it is possible that the parasite culture becomes asynchronous giving the impression of "random" *var* gene expression. Thus the "random" expression of *var* genes in culture may simply represent the asynchronisity of the parasite culture. Greater knowledge of *var* gene expression and the nature of *var* gene switching will hopefully provide answers to these questions .

Analysis of the *var* gene sequences transcribed by 3D7A revealed that the sequences are diverse. Nucleotide homology < 20% between sequences was observed. Such high

sequence diversity may function to maximise the antigenic diversity between PfEMP-1 proteins. Studies have shown that the immune response against the infected erythrocytes surface and presumably PfEMP-1 are important in the development of clinical immunity (Marsh *et al*, 1989, Bull *et al*, 1998; Giha *et al* ., 2000). Antigenic diversity may promote parasite survival by confounding the effects of this host immune response. The sequence diversity and sequence length polymorphisms probably also result in changes in adhesive phenotypes permitting a single parasite to exhibit a number of different adhesive specificities. Alternatively it may be that not all PfEMP-1 variants are involved in adhesion and that disparate sequences only function to create antigenic diversity at the infected erythrocyte surface. Small regions of conserved sequence appear in sequence blocks of between 10-20 amino acids throughout the molecule. This conserved sequences may represent the structural framework of the PfEMP-1 protein. Such conserved sequence blocks have been observed in other malaria antigens (Cooper *et al.,* 1993).

The transcribed *var* gene sequences were analysed alongside *var* gene sequences cloned from a panel of Sudanese clones and a "global" pool of *var* genes from the NCBI genebank database. (Ward *et al.,* 1999). This analysis revealed that although most of the *var* genes sequenced exhibited little homology with *var* genes from other clones, some *var* gene sequences expressed from the 3D7A clone exhibited a high degree of sequence homology with some *var* genes cloned from one of the Sudanese clones 105. The 3D7A*var* clone 149 is identical to the 105 *var* gene SD105J, and 3D7A*Var* 59 and *var* gene SD105M differ by 3 of 335 nucleotides sequenced. At the amino acid level there remains a great deal of sequence identity between some 3D7A *var* genes and some 105 *var* genes. For example the 3D7A *var* clone 37 and 105 clone SD105L are identical at 124 of

125 amino acids and 3D7A clone *var* 43 and SD105R share 128 of 131 amino acids sequenced. The sequence similarity was also observed between 3D7A *var* gene sequences and some of the Brazilian sequences obtained from the genebank database, however the sequence similarities were less marked (<70%)(Ward *et al* 1999).

The similarity of *var* gene sequences from SD 105 and 3D7A may be due to contamination of the 3D7A cultures with SD105 parasites during culture. However during the course of this project 3D7A and the Sudanese parasite SD 105 were never in culture at the same time. No SD105 parasites were cultured during the period of this project, thus eliminating this as a possibility. Another possibility is that 3D7A Stocks have been contaminated with SD 105 parasites. Given the strict control of the WHO Malaria Registry this is also improbable. However this possibility could be completely eliminated by genotyping the cultured parasites using standard methods (Waliker *et al.,* 1987).

One biological explanation for the sequence similarity between parasites from disparate geographies is that there is an exchange of gene sequence during recombination between randomly mating populations. In support of this hypothesis studies have shown that parasites in freely mixing parasite populations undergo recombination in the field (Conway *et al.,* 1999). High rates of homologous recombination have also been observed in laboratory crosses of the parasite (Walliker *et al.,* 1987). Recent data suggests that the diversity observed in *var* genes is greater than what would be expected by homologous recombination alone and that in addition to homologous recombination, gene conversion events between heterologous chromosomes may also be responsible for generating diversity (Freitas-Junior *et al.,* 2000).

These data illustrate that 3D7A transcribes at least 27 *var* genes containing 2 DBL domains DBL-α and DBL-δ. These genes are diverse at the nucleotide level which contributes to the antigenic complexity of the infected erythrocytes surface. These sequences are not clone specific and genes with similar sequence homology have been observed in other parasite clones. These data do not support the contention that the *var* genes expressed by a parasite clone segregate in to distinct non-overlapping repertoires has been suggested previously (Gupta *et al.*, 1994). Rather this indicates that recombination permits exchange of *var* gene sequences between freely mixing genetically distinct parasites.

7.3 PfEMP-1 and cytoadherence

Chapter 3 describes the selection of parasites for adhesion to the surface of CHO cells. The adhesive receptor mediating this phenotype is yet to be described. However this thesis was also concerned with identifying the parasite derived molecule mediating the adhesion of selected 3D7A to the surface of CHO cells.

Binding of infected erythrocytes to the surface of CHO cells was abolished by the treatment of parasites with trypsin. Several laboratories have shown that PfEMP-1 is sensitive to trypsin digestion and that cytoadhesion mediated by PfEMP-1 can be abolished by treatment of parasites with trypsin (Baruch *et al.*, 1996; Gardner *et al.*, 1996). The trypsin sensitivity of this CHO cell adhesive phenotype is consistent with PfEMP-1 being the parasite-derived ligand supporting this adhesive phenotype.

To further analyse the identity of the parasite derived cytoadherence receptor ring stage 3D7A parasites selected for adhesion to CHO cells were subjected to RT-PCR amplification of total RNA using degenerate primers based on *var* genes sequences. The PCR product was cloned and sequenced and found to contain a single *var* gene sequence. Studies have previously shown that selection for a specific adhesive phenotype selects for the transcription of a specific *var* gene, the *var* gene mediating the adhesive phenotype (Rowe *et al.,* 1997; Chen *et al.,* 1998, Reeder *et al.,* 1999, Buffet *et al.,* 1999). It was shown in Chapter 4 that the primers used in the amplification of this gene were capable of amplifying at least 27 *var* genes. Therefore the amplification of a single transcript from the selected 3D7A culture is consistent with the transcription of this gene being selected for during the selection for adhesion and not simply due to bias of the primers used. The trypsin sensitivity of the adhesive phenotype supported by the cloning of a single *var* transcript from the selected parasite culture suggest that this adhesive phenotype is mediated by PfEMP-1. These data support the view that that the *var* gene cloned after selection, *var*-CHO is the gene encoding for the PfEMP-1 protein mediating this adhesion.

It was possible to extend the *var*-CHO sequence using genomics. Sequence reads from the *Plasmodium falciparum* Genome project were used to produce a contiguous sequence from the 5' end of the clone (DBL-β) to the *5'* start codon of the gene. The 3' end of the exon 1 of this *var* gene was however obtained by conventional methods (i.e. cloning and sequencing) because it was not possible to extend the *var*-CHO clone sequence into the *var* gene exon 2 sequence using sequence data from the *P. falciparum* genome project. The first exon of the *var*-CHO gene was sequenced and annotated. The *var*-CHO gene

sequence contains 3DBL domains a DBL-α, DBL-β and a DBL-δ, these domains were defined by sequence motifs specific to each of the DBL domains. The sequence also contains two CIDR domains, CIDR-α and CIDR-β, 3' of DBL-α and DBL-δ respectively. 3' of the DBL-β domain is a small C2 domain which is consistently found in tandem with the DBL-β domain (Smith *et al.,* 2000 b).

PfEMP-1 mediated adhesion is mediated by discrete binding epitopes within specific DBL domains. For example the DBL-α domain is yet to be associated with cytoadherence but has been identified as the domain mediating the rosetting phenotype by two laboratories using different clones which rosette via different host receptors (Rowe *et al.,* 1997; Chen *et al.,* 1998). The CIDR-α domain has been defined as the domain mediating cytoadherence via the membrane glycoprotein CD36 (Baruch *et al.,* 1997) Recently, the tandem PfEMP-1 domain DBL-β -C2 has been identified as the binding domain responsible for adhesion to ICAM-1 (Smith *et al.*, 2000 a). The DBL-γ domain contains the binding epitope mediating cytoadherence to CSA. The presence of the binding domain within the DBL-γ sequence has been confirmed by two laboratories independently using two different parasite clones (Buffet *et al.*, 1999; Reeder *et al.*, 1999). It has been suggested that PfEMP-1 variants that bind to a specific receptor will bind via the same DBL domain. For example it is predicted that all PfEMP-1variants that bind to CSA will bind via a DBL-γ domain (Smith *et al.,* 2000 b). In keeping with this hypothesis the *var*-CHO sequence does not contain a DBL-γ sequence. This provides further support for the view that the 3D7A adherence to CHO cells is not a CSA mediated adhesive phenotype.

To provide conclusive evidence that this adhesive phenotype is PFEMP-1 mediated it

would be necessary to clone fragments or domains of the *var*-CHO gene. Domains of the PfEMP-1 protein can be expressed in bacterial expression vectors as Glutathione-S-transferase (GST) fusion proteins (Baruch *et al.,* 1995, 1997). These fusion proteins can be used directly to inhibit adhesion to the CHO cell surface. This type of experiment has been used in the identification of the CIDR-α as the PfEMP-1 domain of the Malayan camp parasite clone mediating adherence to CD36, and in the identification of the DBL-α domain of the PfEMP-1 from the FCR3 S1.2 clone as the domain mediating rosetting via heparan sulphate (Baruch et al 1997; Chen *et al.,* 1998 a). Another possibility is to raise antibody to specific PfEMP-1 recombinant proteins by the immunisation of mice. The use of these antibodies to inhibit adhesion of cytoadherence provides indirect evidence of the involvement of particular PfEMP-1 domains in adhesion (Reeder *et al.,* 1999). A frequently used method for the identification of adhesive domains within the PfEMP-1 protein has been the cloning of *var* gene sequence into mammalian transfection vectors such as CHO or COS-7 cells. Transfection of *var* sequence into these vectors allows these mammalian cells to support cytoadhesion. This methodology has been used in the identification of the PfEMP-1 domain mediating rosetting via CRI (Rowe *et al.,* 1997) and the PfEMP-1 domains involved in cytoadhernece via CD36, CSA and ICAM-1 (Smith *et al.,* 1998; Buffet *et al.,* 1998; Smith *et al.,* 2000). Identification of the PfEMP-1 domain mediating this adhesive phenotype would permit further investigation on to the molecular basis of this interaction.

7.4 *Var* gene switching

Using RT-PCR dot blot assay it was not possible to detect *var* gene switching in the

parasite clone 3D7A. The possible technical reasons for this have been detailed in chapter 6. One biological explanation for this could be that the parasites, once selected for adhesion to the surface of CHO cells stop switching. However this is inconsistent with the adhesion data which shows that selected 3D7A parasites cultured without selection over a period of 40 generations lose their binding phenotype, presumably due to switching to another PfEMP1 variant. The binding data is consistent with the observations of Roberts *et al.*,(1992), that 3D7A also undergoes antigenic variation, possibly at a rate similar to the A4 parasite clone.

Var genes are located at the subtelomers of each of the 14 *Plasmodium* chromosomes, although the are some chromosome internal *var* gene clusters. The genes are expressed in situ, they are not transported to specific transcription sites to be transcribed (Fischer *et al.*, 1997; Scherf *et al.*, 1998). Recently it has been shown that parasites selected for an specific adhesive phenotype, rosetting, transcribe a number of different *var* genes from different chromosomes simultaneously during early ring stage infection. This transcription, by the early trophozoite stage becomes restricted to the single *var* gene that is translated into protein and expressed at the infected erythrocytes surface (Chen *et al.*, 1998 b). Others have observed a similar pattern of transcription in ring stage parasites when selected for adhesion to CSA. The authors suggest that the multiple transcripts found in the early ring stage disappear as the parasite matures to the trophozoite stage leaving a single transcript. This process has been by described as allelic exclusion (Scherf *et al.*, 1998). The results obtained during the development of this thesis do not support the theory of allelic exclusion. All PCR reactions were conducted on the early, 6-9 hour ring stage, of selected parasites. However only a single transcript was amplified. *Plasmodium* genes are prone to

relaxed gene transcription. It is possible that genes not being transcribed can synthesise short incomplete 5' transcripts at the ring stage of the parasite infection and that multiple transcripts amplified by these authors represent incomplete 5' transcripts, not full length *var* gene transcripts. This idea is supported by Taylor *et al* (1999) who demonstrated by RT-PCR of DBL-α that it is possible to amplify a number of *var* gene transcripts from selected ring stage parasites which when analysed by northern hybridisation, are not complete PfEMP-1 transcripts. Only one full length transcript is present. The RT-PCR data generated in this thesis tend to support this hypothesis however, more experiments on the transcriptional regulation of these genes may help to clarify this issue.

Summary

The work in this thesis has demonstrated that the parasite clone 3D7A adheres to CHO cells by an undefined cytoadherence receptor. This receptor must be identified as it may represent an adhesion molecule involved in the pathogenesis of cerebral and/or placental malaria. We have also demonstrated that consistent with the findings of others, the adhesive phenotype exhibited by 3D7A is mediated by the parasite derived protein PfEMP-1.Conformation of this requires recombinant protein expression studies, which will facilitate the identification of which epitope from the var-CHO sequence is involved in adhesion. This will also permit immunological studies investigating the clinical relevance of the immune response against this adhesive epitope by determining whether the immune response against this epitope is protective against disease or infection. Further studies will also determine whether this epitope can be used to prevent adhesion of parasites to the adhesion molecule expressed at the surface of CHO cell, experiments which may have implications for anti adhesive therapy. These studies should form the basis for further experimentation based on the work described in this thesis.

Bibliography

Adams, J. H., Sim, B. K., Dolan, S. A., Fang, X., Kaslow, D. C., and Miller, L. H. (1992). A family of erythrocyte binding proteins of malaria parasites. Proc Natl Acad Sci U S A *89*, 7085-9.

Aguiar, J. C., Albrecht, G. R., Cegielski, P., Greenwood, B. M., Jensen, J. B., Lallinger, G., Martinez, A., McGregor, I. A., Minjas, J. N., Neequaye, J., and et al. (1992). Agglutination of *Plasmodium falciparum*-infected erythrocytes from east and west African isolates by human sera from distant geographic regions. Am J Trop Med Hyg *47*, 621-32.

Allan, R. J., Rowe, A., and Kwiatkowski, D. (1993). *Plasmodium falciparum* varies in its ability to induce tumor necrosis factor. Infect Immun *61*, 4772-6.

Arnberg, N., Edlund, K., Kidd, A. H., and Wadell, G. (2000). Adenovirus type 37 uses sialic acid as a cellular receptor. J Virol *74*, 42-8.

Barnwell, J. W., Asch, A. S., Nachman, R. L., Yamaya, M., Aikawa, M., and Ingravallo, P. (1989). A human 88-kD membrane glycoprotein (CD36) functions in vitro as a receptor for a cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes. J Clin Invest *84*, 765-72.

Barnwell, J. W., Howard, R. J., Coon, H. G., and Miller, L. H. (1983). Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. Infect Immun *40*, 985-94.

Baruch, D. I., Gormely, J. A., Ma, C., Howard, R. J., and Pasloske, B. L. (1996). *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. Proc Natl Acad Sci U S A *93*, 3497-502.

Baruch, D. I., Ma, X. C., Singh, H. B., Bi, X., Pasloske, B. L., and Howard, R. J. (1997). Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum*

infected erythrocytes to CD36: conserved function with variant sequence. Blood *90*, 3766-75.

Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F., and Howard, R. J. (1995). Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes .Cell *82*, 77-87.

Beaudoin, R. L., Strome, C. P., Mitchell, F., and Tubergen, T. A. (1977). *Plasmodium berghei*: immunization of mice against the ANKA strain using the unaltered sporozoite as an antigen. Exp Parasitol *42*, 1-5.

Beeson, J. G., Rogerson, S. J., Cooke, B. M., Reeder, J. C., Chai, W., Lawson, A. M., Molyneux, M. E., and Brown, G. V. (2000). Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria [see comments]. Nat Med *6*, 86-90.

Berendt, A. R., Ferguson, D. J., Gardner, J., Turner, G., Rowe, A., McCormick, C., Roberts, D., Craig, A., Pinches, R., Elford, B. C., and et al. (1994). Molecular mechanisms of sequestration in malaria. Parasitology *108*, S19-28.

Berendt, A. R., McDowall, A., Craig, A. G., Bates, P. A., Sternberg, M. J., Marsh, K., Newbold, C. I., and Hogg, N. (1992). The binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. Cell *68*, 71-81.

Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I., and Marsh, K. (1989). Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature *341*, 57-9.

Biggs, B. A., Anders, R. F., Dillon, H. E., Davern, K. M., Martin, M., Petersen, C., and Brown, G. V. (1992). Adherence of infected erythrocytes to venular endothelium selects for antigenic variants of *Plasmodium falciparum*. J Immunol *149*, 2047-54.

Biggs, B. A., Gooze, L., Wycherley, K., Wilkinson, D., Boyd, A. W., Forsyth, K. P., Edelman, L., Brown, G. V., and Leech, J. H. (1990). Knob-independent cytoadherence

of *Plasmodium falciparum* to the leukocyte differentiation antigen CD36. J Exp Med *171*, 1883-92.

Biggs, B. A., Gooze, L., Wycherley, K., Wollish, W., Southwell, B., Leech, J. H., and Brown, G. V. (1991). Antigenic variation in *Plasmodium falciparum*. Proc Natl Acad Sci U S A *88*, 9171-4.

Blackman, M. J., Scott-Finnigan, T. J., Shai, S., and Holder, A. A. (1994). Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J Exp Med *180*, 389-93.

Bloland, P., Slutsker, L., Steketee, R. W., Wirima, J. J., Heymann, D. L., and Breman, J. G. (1996). Rates and risk factors for mortality during the first two years of life in rural Malawi. Am J Trop Med Hyg *55*, 82-6.

Borst, P., Bitter, W., McCulloch, R., Van Leeuwen, F., and Rudenko, G. (1995). Antigenic variation in malaria . Cell *82*, 1-4.

Bowman, S., Lawson, D., Basham, D., Brown, D., Chillingworth, T., Churcher, C. M., Craig, A., Davies, R. M., Devlin, K., Feltwell, T., Gentles, S., Gwilliam, R., Hamlin, N., Harris, D., Holroyd, S., Hornsby, T., Horrocks, P., Jagels, K., Jassal, B., Kyes, S., McLean, J., Moule, S., Mungall, K., Murphy, L., Barrell, B. G., and et al. (1999). The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum* . Nature *400*, 532-8.

Brabin, B. (1991). An assessment of low birthweight risk in primiparae as an indicator of malaria control in pregnancy. Int J Epidemiol *20*, 276-83.

Brabin, B. J. (1983). An analysis of malaria in pregnancy in Africa. Bull World Health Organ *61*, 1005-16.

Brown, K. N., and Brown, I. N. (1965). Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. Nature *208*, 1286-8.

Brown, K. N., Brown, I. N., Phillips, R. S., Trigg, P. I., Hills, L. A., Wolstencroft, R. A., and Dumonde, D. C. (1970). Immunity to malaria: studies with *Plasmodium knowlesi.* Trans R Soc Trop Med Hyg *64*, 3-5.

Bruce-Chwatt (1952). Malaria in African infants and children in Southeren Nigeria. Annals of tropical medicine and parasitology *46*, 173-200.

Buffet, P. A., Gamain, B., Scheidig, C., Baruch, D., Smith, J. D., Hernandez-Rivas, R., Pouvelle, B., Oishi, S., Fujii, N., Fusai, T., Parzy, D., Miller, L. H., Gysin, J., and Scherf, A. (1999). *Plasmodium falciparum* domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. Proc Natl Acad Sci U S A *96*, 12743-8.

Bull, P. C., Lowe, B. S., Kortok, M., and Marsh, K. (1999). Antibody recognition of *Plasmodium falciparum* erythrocyte surface antigens in Kenya: evidence for rare and prevalent variants. Infect Immun *67*, 733-9.

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I., and Marsh, K. (1998). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat Med *4*, 358-60.

Bulmer, J. N., Rasheed, F. N., Francis, N., Morrison, L., and Greenwood, B. M. (1993). Placental malaria. I. Pathological classification. Histopathology *22*, 211-8.

Butcher, G. A. (1992). HIV and Malaria: a lesson in immunology. Parasitology Today *8*, 307-311.

Carlson, J., Helmby, H., Hill, A. V., Brewster, D., Greenwood, B. M., and Wahlgren, M. (1990). Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. Lancet *336*, 1457-60.

Carlson, J., and Wahlgren, M. (1992). *Plasmodium falciparum* erythrocyte rosetting is mediated by promiscuous lectin-like interactions. J Exp Med *176*, 1311-7.

Cerami, C., Frevert, U., Sinnis, P., Takacs, B., Clavijo, P., Santos, M. J., and Nussenzweig, V. (1992). The basolateral domain of the hepatocyte plasma membrane

bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. Cell *70*, 1021-33.

Chaiyaroj, S. C., Angkasekwinai, P., Buranakiti, A., Looareesuwan, S., Rogerson, S. J., and Brown, G. V. (1996). Cytoadherence characteristics of *Plasmodium falciparum* isolates from Thailand: evidence for chondroitin sulfate a as a cytoadherence receptor. Am J Trop Med Hyg *55*, 76-80.

Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S., and Wahlgren, M. (1998a). Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite P. falciparum. J Exp Med *187*, 15-23.

Chen, Q., Fernandez, V., Sundstrom, A., Schlichtherle, M., Datta, S., Hagblom, P., and Wahlgren, M. (1998b). Developmental selection of var gene expression in *Plasmodium falciparum*. Nature *394*, 392-5.

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., Lanzer, M., and Saul, A. (1998). stevor and rif are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. Mol Biochem Parasitol *97*, 161-76.

Chulay, J. D., and Ockenhouse, C. F. (1990). Host receptors for malaria-infected erythrocytes. Am J Trop Med Hyg *43*, 6-14.

Clough, B., Atilola, F. A., and Pasvol, G. (1998). The role of rosetting in the multiplication of *Plasmodium falciparum*: rosette formation neither enhances nor targets parasite invasion into uninfected red cells. Br J Haematol *100*, 99-104.

Cooke, B. M., Berendt, A. R., Craig, A. G., MacGregor, J., Newbold, C. I., and Nash, G. B. (1994). Rolling and stationary cytoadhesion of red blood cells parasitized by *Plasmodium falciparum*: separate roles for ICAM-1, CD36 and thrombospondin. Br J Haematol *87*, 162-70.

Cooper, J. A. (1993). Merozoite surface antigen-1 of *Plasmodium*. Parasitology Today *9*, 50-55.

Cot, M., Roisin, A., Barro, D., Yada, A., Verhave, J. P., Carnevale, P., and Breart, G. (1992). Effect of chloroquine chemoprophylaxis during pregnancy on birth weight: results of a randomized trial. Am J Trop Med Hyg *46*, 21-7.

Covell, G. N., W. D (1951). Clinical chemotheraputic and immunological studies on induced malaria. British Medical Bullitin *8*, 51-55.

Crabb, B. S., Cooke, B. M., Reeder, J. C., Waller, R. F., Caruana, S. R., Davern, K. M., Wickham, M. E., Brown, G. V., Coppel, R. L., and Cowman, A. F. (1997). Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell *89*, 287-96.

Crandall, I., and Sherman, I. W. (1991). *Plasmodium falciparum* (human malaria)-induced modifications in human erythrocyte band 3 protein. Parasitology *102 Pt 3*, 335-40.

Crutchfield, K. L., Shinde Patil, V. R., Campbell, C. J., Parkos, C. A., Allport, J. R., and Goetz, D. J. (2000). CD11b/CD18-coated microspheres attach to E-selectin under flow. J Leukoc Biol *67*, 196-205.

David, P. H., Handunnetti, S. M., Leech, J. H., Gamage, P., and Mendis, K. N. (1988). Rosetting: a new cytoadherence property of malaria-infected erythrocytes. Am J Trop Med Hyg *38*, 289-97.

David, P. H., Hommel, M., Miller, L. H., Udeinya, I. J., and Oligino, L. D. (1983). Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. Proc Natl Acad Sci U S A *80*, 5075-9.

Doolan, D. L., Houghten, R. A., and Good, M. F. (1991). Location of human cytotoxic T cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein. Int Immunol *3*, 511-6.

Duffy, P. E., and Fried, M. (1999). Malaria during pregnancy: parasites, antibodies and chondroitin sulphate A. Biochem Soc Trans *27*, 478-82.

Eaton, M. D. (1938). The agglutination of *P. Knowelsi* by immune serum. Journal Of experimental Medicine *67*, 857-69.

Egan, A. F., Morris, J., Barnish, G., Allen, S., Greenwood, B. M., Kaslow, D. C., Holder, A. A., and Riley, E. M. (1996). Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. J Infect Dis *173*, 765-9.

Enea, V., Ellis, J., Zavala, F., Arnot, D. E., Asavanich, A., Masuda, A., Quakyi, I., and Nussenzweig, R. S. (1984). DNA cloning of *Plasmodium falciparum* circumsporozoite gene: amino acid sequence of repetitive epitope. Science *225*, 628-30.

Esko, J. D. (1991). Genetic analysis of proteoglycan structure, function and metabolism. Curr Opin Cell Biol *3*, 805-16.

Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985). Animal cell mutants defective in glycosaminoglycan biosynthesis. Proc Natl Acad Sci U S A *82*, 3197-201.

Fernandez, V., Hommel, M., Chen, Q., Hagblom, P., and Wahlgren, M. (1999). Small, clonally variant antigens expressed on the surface of the *Plasmodium falciparum*-infected erythrocyte are encoded by the rif gene family and are the target of human immune responses. J Exp Med *190*, 1393-404.

Fernandez-Reyes, D., Craig, A. G., Kyes, S. A., Peshu, N., Snow, R. W., Berendt, A. R., Marsh, K., and Newbold, C. I. (1997). A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya. Hum Mol Genet *6*, 1357-60.

Fischer, K., Horrocks, P., Preuss, M., Wiesner, J., Wunsch, S., Camargo, A. A., and Lanzer, M. (1997). Expression of var genes located within polymorphic subtelomeric domains of *Plasmodium falciparum* chromosomes. Mol Cell Biol *17*, 3679-86.

Forsyth, K. P., Philip, G., Smith, T., Kum, E., Southwell, B., and Brown, G. V. (1989). Diversity of antigens expressed on the surface of erythrocytes infected with mature *Plasmodium falciparum* parasites in Papua New Guinea. Am J Trop Med Hyg *41*, 259-65.

Freitas-Junior, L. H., Bottius, E., Pirrit, L. A., Deitsch, K. W., Scheidig, C., Guinet, F., Nehrbass, U., Wellems, T. E., and Scherf, A. (2000). Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum* . Nature *407*, 1018-22.

Fried, M., and Duffy, P. E. (1996). Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta . Science *272*, 1502-4.

Fried, M., and Duffy, P. E. (1998). Maternal malaria and parasite adhesion. J Mol Med *76*, 162-71.

Galbraith, R. M., Fox, H., Hsi, B., Galbraith, G. M., Bray, R. S., and Faulk, W. P. (1980). The human materno-foetal relationship in malaria. II. Histological, ultrastructural and immunopathological studies of the placenta. Trans R Soc Trop Med Hyg *74*, 61-72.

Gardner, J. P., Pinches, R. A., Roberts, D. J., and Newbold, C. I. (1996). Variant antigens and endothelial receptor adhesion in *Plasmodium falciparum*. Proc Natl Acad Sci U S A *93*, 3503-8.

Gardner, M. J., Tettelin, H., Carucci, D. J., Cummings, L. M., Aravind, L., Koonin, E. V., Shallom, S., Mason, T., Yu, K., Fujii, C., Pederson, J., Shen, K., Jing, J., Aston, C., Lai, Z., Schwartz, D. C., Pertea, M., Salzberg, S., Zhou, L., Sutton, G. G., Clayton, R., White, O., Smith, H. O., Fraser, C. M., Hoffman, S. L., and et al. (1998). Chromosome 2 sequence of the human malaria parasite *Plasmodium falciparum* [published erratum appears in Science 1998 Dec 4;282(5395):1827]. Science *282*, 1126-32.

Giha, H. A., Staalsoe, T., Dodoo, D., Roper, C., Satti, G. M., Arnot, D. E., Hviid, L., and Theander, T. G. (2000). Antibodies to variable *Plasmodium falciparum*-infected erythrocyte surface antigens are associated with protection from novel malaria infections. Immunol Lett *71*, 117-26.

Giha, H. A., Theander, T. G., Staalso, T., Roper, C., Elhassan, I. M., Babiker, H., Satti, G. M., Arnot, D. E., and Hviid, L. (1998). Seasonal variation in agglutination of *Plasmodium falciparum*-infected erythrocytes. Am J Trop Med Hyg *58*, 399-405.

Gilbert, S. C., Plebanski, M., Gupta, S., Morris, J., Cox, M., Aidoo, M., Kwiatkowski, D., Greenwood, B. M., Whittle, H. C., and Hill, A. V. (1998). Association of malaria parasite population structure, HLA, and immunological antagonism Science *279*, 1173-7.

Gilles, H. M., Lawson, J. B., Sibellas, M., Voller, A., and Allan, N. (1969). Malaria and pregnancy. Trans R Soc Trop Med Hyg *63*, 1.

Goldring, J. D., and Hommel, M. (1992). Variation in the cytoadherence characteristics of malaria parasites: is this a true virulence factor? Mem Inst Oswaldo Cruz *87*, 313-22.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J Mol Biol *166*, 557-80.

Handunnetti, S. M., Mendis, K. N., and David, P. H. (1987). Antigenic variation of cloned Plasmodium fragile in its natural host Macaca sinica. Sequential appearance of successive variant antigenic types. J Exp Med *165*, 1269-83.

Hasler, T., Albrecht, G. R., Van Schravendijk, M. R., Aguiar, J. C., Morehead, K. E., Pasloske, B. L., Ma, C., Barnwell, J. W., Greenwood, B., and Howard, R. J. (1993). An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. Am J Trop Med Hyg *48*, 332-47.

Herrington, D., Davis, J., Nardin, E., Beier, M., Cortese, J., Eddy, H., Losonsky, G., Hollingdale, M., Sztein, M., Levine, M., and et al. (1991). Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. Am J Trop Med Hyg *45*, 539-47.

Hill, A. V., Elvin, J., Willis, A. C., Aidoo, M., Allsopp, C. E., Gotch, F. M., Gao, X. M., Takiguchi, M., Greenwood, B. M., Townsend, A. R., and et al. (1992). Molecular analysis of the association of HLA-B53 and resistance to severe malaria .Nature *360*, 434-9.

Ho, M., Schollaardt, T., Niu, X., Looareesuwan, S., Patel, K. D., and Kubes, P. (1998). Characterization of *Plasmodium falciparum*-infected erythrocyte and P-selectin interaction under flow conditions. Blood *91*, 4803-9.

Ho, M., Singh, B., Looareesuwan, S., Davis, T. M., Bunnag, D., and White, N. J. (1991). Clinical correlates of in vitro *Plasmodium falciparum* cytoadherence. Infect Immun *59*, 873-8.

Hommel, M., David, P. H., and Oligino, L. D. (1983). Surface alterations of erythrocytes in *Plasmodium falciparum* malaria. Antigenic variation, antigenic diversity, and the role of the spleen. J Exp Med *157*, 1137-48.

Howard, R. J., Barnwell, J. W., and Kao, V. (1983). Antigenic variation of Plasmodium knowlesi malaria: identification of the variant antigen on infected erythrocytes. Proc Natl Acad Sci U S A *80*, 4129-33.

Howard, R. J., Lyon, J. A., Diggs, C. L., Haynes, J. D., Leech, J. H., Barnwell, J. W., Aley, S. B., Aikawa, M., and Miller, L. H. (1984). Localization of the major *Plasmodium falciparum* glycoprotein on the surface of mature intraerythrocytic trophozoites and schizonts. Mol Biochem Parasitol *11*, 349-62.

Hyde, J. (1993). Protocols in molecular biology. Methods in molecular Biology, 181-210.

Jeffery, G. M. (1966). Epdemiological significance of repeated infections with homologous and heterologous strains and species of Plasmodium. Bullitin of the World Health Organisation *35*, 873-82.

Johnston, B., Chee, A., Issekutz, T. B., Ugarova, T., Fox-Robichaud, A., Hickey, M. J., and Kubes, P. (2000). Alpha 4 integrin-dependent leukocyte recruitment does not require VCAM-1 in a chronic model of inflammation. J Immunol *164*, 3337-44.

Kaul, D. K., Roth, E. F., Jr., Nagel, R. L., Howard, R. J., and Handunnetti, S. M. (1991). Rosetting of *Plasmodium falciparum*-infected red blood cells with uninfected red blood cells enhances microvascular obstruction under flow conditions. Blood *78*, 812-9.

Kilejian, A. (1979). Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with *Plasmodium falciparum*. Proc Natl Acad Sci U S A *76*, 4650-3.

Kumar, S., Miller, L. H., Quakyi, I. A., Keister, D. B., Houghten, R. A., Maloy, W. L., Moss, B., Berzofsky, J. A., and Good, M. F. (1988). Cytotoxic T cells specific for the circumsporozoite protein of *Plasmodium falciparum*. Nature *334*, 258-60.

Kwiatkowski, D. (1995). Malaria toxins and the regulation of parasite density. Parasitology Today *11*, 206-212.

Kwiatkowski, D., Hill, A. V., Sambou, I., Twumasi, P., Castracane, J., Manogue, K. R., Cerami, A., Brewster, D. R., and Greenwood, B. M. (1990). TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet *336*, 1201-4.

Kyes, S., Taylor, H., Craig, A., Marsh, K., and Newbold, C. (1997). Genomic representation of var gene sequences in *Plasmodium falciparum* field isolates from different geographic regions [published erratum appears in Mol Biochem Parasitol 1998 May 15;93(1):159]. Mol Biochem Parasitol *87*, 235-8.

Kyes, S. A., Rowe, J. A., Kriek, N., and Newbold, C. I. (1999). Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum*. Proc Natl Acad Sci U S A *96*, 9333-8.

Langhorne, J., Gillard, S., Simon, B., Slade, S., and Eichmann, K. (1989). Frequencies of CD4+ T cells reactive with Plasmodium chabaudi chabaudi: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. Int Immunol *1*, 416-24.

Laveran A (1880). Note sur un nouveau parasite trouve dans le sang du plusieurs malades attints de fievre palustre. Bullitin de l'Academie Nationale de Medicine (paris) *9*, 1235.

Leech, J. H., Barnwell, J. W., Miller, L. H., and Howard, R. J. (1984). Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. J Exp Med *159*, 1567-75.

Lucas, J. Z., and Sherman, I. W. (1998). *Plasmodium falciparum*: thrombospondin mediates parasitized erythrocyte band 3-related adhesin binding. Exp Parasitol *89*, 78-85.

MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S., and Warrell, D. A. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol *119*, 385-401.

Maitland, K., Williams, T. N., Bennett, S., Newbold, C. I., Peto, T. E., Viji, J., Timothy, R., Clegg, J. B., Weatherall, D. J., and Bowden, D. K. (1996). The interaction between *Plasmodium falciparum* and P. vivax in children on Espiritu Santo island, Vanuatu. Trans R Soc Trop Med Hyg *90*, 614-20.

Makrides, S. C., Scesney, S. M., Ford, P. J., Evans, K. S., Carson, G. R., and Marsh, H. C., Jr. (1992). Cell surface expression of the C3b/C4b receptor (CR1) protects Chinese hamster ovary cells from lysis by human complement. J Biol Chem *267*, 24754-61.

Marsh, K. (1992). Malaria--a neglected disease? Parasitology *104*, S53-69.

Marsh, K., and Howard, R. J. (1986). Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. Science *231*, 150-3.

Marsh, K., Marsh, V. M., Brown, J., Whittle, H. C., and Greenwood, B. M. (1988). *Plasmodium falciparum*: the behavior of clinical isolates in an in vitro model of infected red blood cell sequestration. Exp Parasitol *65*, 202-8.

Marsh, K., Otoo, L., Hayes, R. J., Carson, D. C., and Greenwood, B. M. (1989). Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans R Soc Trop Med Hyg *83*, 293-303.

Matteelli, A., Donato, F., Shein, A., Muchi, J. A., Abass, A. K., Mariani, M., Leopardi, O., Maxwell, C. A., and Carosi, G. (1996). Malarial infection and birthweight in urban Zanzibar, Tanzania. Ann Trop Med Parasitol *90*, 125-34.

McCormick, C. J., Craig, A., Roberts, D., Newbold, C. I., and Berendt, A. R. (1997). Intercellular adhesion molecule-1 and CD36 synergize to mediate adherence of *Plasmodium falciparum*-infected erythrocytes to cultured human microvascular endothelial cells. J Clin Invest *100*, 2521-9.

McDonald, V., and Phillips, R. S. (1980). *Plasmodium chabaudi*: adoptive transfer of immunity with different spleen cell populations and development of protective activity in the serum of lethally irradiated recipient mice. Exp Parasitol *49*, 26-33.

McGregor, I. A. (1984). Epidemiology, malaria and pregnancy. Am J Trop Med Hyg *33*, 517-25.

McGregor, I. A., Wilson, M. E., and Billewicz, W. Z. (1983). Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birthweight and placental weight. Trans R Soc Trop Med Hyg *77*, 232-44.

Miller, L. H. (1969). Distribution of mature trophozoites and schizonts of *Plasmodium falciparum* in the organs of Aotus trivirgatus, the night monkey. Am J Trop Med Hyg *18*, 860-5.

Ming, M., Chuenkova, M., Ortega-Barria, E., and Pereira, M. E. (1993). Mediation of *Trypanosoma cruzi* invasion by sialic acid on the host cell and trans-sialidase on the trypanosome. Mol Biochem Parasitol *59*, 243-52.

Nash, G. B., Cooke, B. M., Marsh, K., Berendt, A., Newbold, C., and Stuart, J. (1992). Rheological analysis of the adhesive interactions of red blood cells parasitized by *Plasmodium falciparum*. Blood *79*, 798-807.

Newbold, C., Warn, P., Black, G., Berendt, A., Craig, A., Snow, B., Msobo, M., Peshu, N., and Marsh, K. (1997b). Receptor-specific adhesion and clinical disease in *Plasmodium falciparum* [see comments]. Am J Trop Med Hyg *57*, 389-98.

Newbold, C. I., Craig, A. G., Kyes, S., Berendt, A. R., Snow, R. W., Peshu, N., and Marsh, K. (1997a). PfEMP1, polymorphism and pathogenesis. Ann Trop Med Parasitol *91*, 551-7.

Ockenhouse, C. F., Ho, M., Tandon, N. N., Van Seventer, G. A., Shaw, S., White, N. J., Jamieson, G. A., Chulay, J. D., and Webster, H. K. (1991b). Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria: differential adhesion of infected erythrocytes to CD36 and ICAM-1. J Infect Dis *164*, 163-9.

Ockenhouse, C. F., Klotz, F. W., Tandon, N. N., and Jamieson, G. A. (1991a). Sequestrin, a CD36 recognition protein on *Plasmodium falciparum* malaria-infected erythrocytes identified by anti-idiotype antibodies. Proc Natl Acad Sci U S A *88*, 3175-9.

Ockenhouse, C. F., Tegoshi, T., Maeno, Y., Benjamin, C., Ho, M., Kan, K. E., Thway, Y., Win, K., Aikawa, M., and Lobb, R. R. (1992). Human vascular endothelial cell adhesion receptors for *Plasmodium falciparum*-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1. J Exp Med *176*, 1183-9.

Oquendo, P., Hundt, E., Lawler, J., and Seed, B. (1989). CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitized erythrocytes. Cell *58*, 95-101.

Ordi, J., Ismail, M. R., Ventura, P. J., Kahigwa, E., Hirt, R., Cardesa, A., Alonso, P. L., and Menendez, C. (1998). Massive chronic intervillositis of the placenta associated with malaria infection. Am J Surg Pathol *22*, 1006-11.

Orjih, A. U., and Nussenzweig, R. S. (1980). Immunization against rodent malaria with cryopreserved irradiated sporozoites of Plasmodium berghei. Am J Trop Med Hyg *29*, 343-7.

Pober, J. S. (1988). TNF as an activator of vascular endothelium. Ann Inst Pasteur Immunol *139*, 317-23.

Pouvelle, B., Buffet, P. A., Lepolard, C., Scherf, A., and Gysin, J. (2000). Cytoadhesion of *plasmodium falciparum* ring-stage-infected erythrocytes . Nat Med *6*, 1264-8.

Pouvelle, B., Fusai, T., and Gysin, J. (1998). [*Plasmodium falciparum* and chondroitin-4-sulfate: the new key couple in sequestration]. Med Trop *58*, 187-98.

Rainger, G. E., Buckley, C., Simmons, D. L., and Nash, G. B. (1997). Cross-talk between cell adhesion molecules regulates the migration velocity of neutrophils. Curr Biol *7*, 316-25.

Raventos-Suarez, C., Kaul, D. K., Macaluso, F., and Nagel, R. L. (1985). Membrane knobs are required for the microcirculatory obstruction induced by *Plasmodium falciparum*-infected erythrocytes. Proc Natl Acad Sci U S A *82*, 3829-33.

Reeder, J. C., and Brown, G. V. (1996). Antigenic variation and immune evasion in *Plasmodium falciparum* malaria. Immunol Cell Biol *74*, 546-54.

Reeder, J. C., Cowman, A. F., Davern, K. M., Beeson, J. G., Thompson, J. K., Rogerson, S. J., and Brown, G. V. (1999). The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by P. falciparum erythrocyte membrane protein 1. Proc Natl Acad Sci U S A *96*, 5198-202.

Reeder, J. C., Rogerson, S. J., al-Yaman, F., Anders, R. F., Coppel, R. L., Novakovic, S., Alpers, M. P., and Brown, G. V. (1994). Diversity of agglutinating phenotype, cytoadherence, and rosette-forming characteristics of *Plasmodium falciparum* isolates from Papua New Guinean children. Am J Trop Med Hyg *51*, 45-55.

Ricke, C. H., Staalsoe, T., Koram, K., Akanmori, B. D., Riley, E. M., Theander, T. G., and Hviid, L. (2000). Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. J Immunol *165*, 3309-16.

Ringwald, P., Peyron, F., Lepers, J. P., Rabarison, P., Rakotomalala, C., Razanamparany, M., Rabodonirina, M., Roux, J., and Le Bras, J. (1993). Parasite virulence factors during falciparum malaria: rosetting, cytoadherence, and modulation of cytoadherence by cytokines. Infect Immun *61*, 5198-204.

Roberts, D. D., Sherwood, J. A., Spitalnik, S. L., Panton, L. J., Howard, R. J., Dixit, V. M., Frazier, W. A., Miller, L. H., and Ginsburg, V. (1985). Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence. Nature *318*, 64-6.

Roberts, D. J., Craig, A. G., Berendt, A. R., Pinches, R., Nash, G., Marsh, K., and Newbold, C. I. (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria . Nature *357*, 689-92.

Roberts, D. J., Pain, A., Kai, O., Kortok, M., and Marsh, K. (2000). Autoagglutination of malaria-infected red blood cells and malaria severity .Lancet *355*, 1427-8.

Robertson, S. A., Seamark, R. F., Guilbert, L. J., and Wegmann, T. G. (1994). The role of cytokines in gestation. Crit Rev Immunol *14*, 239-92.

Rogerson, S. J., and Beeson, J. G. (1999 b). The placenta in malaria: mechanisms of infection, disease and foetal morbidity. Ann Trop Med Parasitol *93 Suppl 1*, S35-42.

Rogerson, S. J., Chaiyaroj, S. C., Ng, K., Reeder, J. C., and Brown, G. V. (1995). Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. J Exp Med *182*, 15-20.

Rogerson, S. J., Tembenu, R., Dobano, C., Plitt, S., Taylor, T. E., and Molyneux, M. E. (1999 a). Cytoadherence characteristics of *Plasmodium falciparum*-infected erythrocytes from Malawian children with severe and uncomplicated malaria. Am J Trop Med Hyg *61*, 467-72.

Rothlein, R., Czajkowski, M., O'Neill, M. M., Marlin, S. D., Mainolfi, E., and Merluzzi, V. J. (1988). Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. J Immunol *141*, 1665-9.

Rowe, J. A., Moulds, J. M., Newbold, C. I., and Miller, L. H. (1997). *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. Nature *388*, 292-5.

Rubio, J. P., Thompson, J. K., and Cowman, A. F. (1996). The var genes of *Plasmodium falciparum* are located in the subtelomeric region of most chromosomes. Embo J *15*, 4069-77.

Saiki, R. K., Chang, C. A., Levenson, C. H., Warren, T. C., Boehm, C. D., Kazazian, H. H., Jr., and Erlich, H. A. (1988). Diagnosis of sickle cell anemia and beta-thalassemia with enzymatically amplified DNA and nonradioactive allele-specific oligonucleotide probes. N Engl J Med *319*, 537-41.

Sambrook, J. F., E. F and Maniatis, T (1989). Molecular cloning: a laboroatory Manual. Cold Spring Harbour Laboratory Press, 612-631.

Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatar, C., Pouvelle, B., Gysin, J., and Lanzer, M. (1998). Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in *Plasmodium falciparum*. EMBO J *17*, 5418-26.

Sedegah, M., Sim, B. K., Mason, C., Nutman, T., Malik, A., Roberts, C., Johnson, A., Ochola, J., Koech, D., Were, B., and et al. (1992). Naturally acquired CD8+ cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. J Immunol *149*, 966-71.

Shortt H E, G. P. C. C. (1948). Demonstration of a persisting erythrocytic cycle of Plasmodium cynomologi and its bearing on the production of relapses. British Medical Journal *1*, 1225-1228.

Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I., and Miller, L. H. (1995). Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell *82*, 101-10.

Smith, J. D., Craig, A. G., Kriek, N., Hudson-Taylor, D., Kyes, S., Fagen, T., Pinches, R., Baruch, D. I., Newbold, C. I., and Miller, L. H. (2000a). Identification of a *Plasmodium falciparum* intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. Proc Natl Acad Sci U S A *97*, 1766-71.

Smith, J. D., Kyes, S., Craig, A. G., Fagan, T., Hudson-Taylor, D., Miller, L. H., Baruch, D. I., and Newbold, C. I. (1998). Analysis of adhesive domains from the A4VAR *Plasmodium falciparum* erythrocyte membrane protein-1 identifies a CD36 binding domain. Mol Biochem Parasitol *97*, 133-48.

Smith, J. D., Subramanian, G., Gamain, B., Baruch, D. I., and Miller, L. H. (2000b). Classification of adhesive domains in the *Plasmodium falciparum* erythrocyte membrane protein 1 family . Mol Biochem Parasitol *110*, 293-310.

Steketee, R. W., Wirima, J. J., and Campbell, C. C. (1996). Developing effective strategies for malaria prevention programs for pregnant African women. Am J Trop Med Hyg *55*, 95-100.

Stewart, M. J., Nawrot, R. J., Schulman, S., and Vanderberg, J. P. (1986). *Plasmodium berghei* sporozoite invasion is blocked in vitro by sporozoite-immobilizing antibodies. Infect Immun *51*, 859-64.

Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., Ravetch, J. A., and Wellems, T. E. (1995). The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. Cell *82*, 89-100.

Taylor, H. M., Kyes, S. A., Harris, D., Kriek, N., and Newbold, C. I. (2000). A study of var gene transcription in vitro using universal *var* gene primers. Mol Biochem Parasitol *105*, 13-23.

Taylor-Robinson, A. W., and Phillips, R. S. (1994b). B cells are required for the switch from Th1- to Th2-regulated immune responses to *Plasmodium chabaudi chabaudi* infection. Infect Immun *62*, 2490-8.

Taylor-Robinson, A. W., and Phillips, R. S. (1993). Protective CD4+ T-cell lines raised against *Plasmodium chabaudi* show characteristics of either Th1 or Th2 cells. Parasite Immunol *15*, 301-10.

Taylor-Robinson, A. W., and Phillips, R. S. (1994a). Th1 and Th2 CD4+ T cell clones specific for *Plasmodium chabaudi* but not for an unrelated antigen protect against blood stage P. chabaudi infection. Eur J Immunol *24*, 158-64.

Trager, W., and Jensen, J. B. (1976). Human malaria parasites in continuous culture. Science *193*, 673-5.

Treutiger, C. J., Heddini, A., Fernandez, V., Muller, W. A., and Wahlgren, M. (1997). PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes . Nat Med *3*, 1405-8.

Turner, G. D., Morrison, H., Jones, M., Davis, T. M., Looareesuwan, S., Buley, I. D., Gatter, K. C., Newbold, C. I., Pukritayakamee, S., Nagachinta, B., and et al. (1994). An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am J Pathol *145*, 1057-69.

Udeinya, I. J., and Akogyeram, C. O. (1993). Induction of adhesiveness in human endothelial cells by *Plasmodium falciparum*-infected erythrocytes. Am J Trop Med Hyg *48*, 488-95.

Udeinya, I. J., Schmidt, J. A., Aikawa, M., Miller, L. H., and Green, I. (1981). *Falciparum* malaria-infected erythrocytes specifically bind to cultured human endothelial cells. Science *213*, 555-7.

Udomsangpetch, R., Aikawa, M., Berzins, K., Wahlgren, M., and Perlmann, P. (1989). Cytoadherence of knobless *Plasmodium falciparum*-infected erythrocytes and its inhibition by a human monoclonal antibody . Nature *338*, 763-5.

Urban, B. C., Ferguson, D. J., Pain, A., Willcox, N., Plebanski, M., Austyn, J. M., and Roberts, D. J. (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells . Nature *400*, 73-7.

Vanmaele, R. P., Finlayson, M. C., and Armstrong, G. D. (1995). Effect of enteropathogenic Escherichia coli on adherent properties of Chinese hamster ovary cells. Infect Immun *63*, 191-8.

Walter, P., Garin, J. F., Blot, P., and Philippe, E. (1981). [The placenta and malaria. A morphologic, parasitologic and clinical study (author's transl)]. J Gynecol Obstet Biol Reprod *10*, 535-42.

Ward, C. P., Clottey, G. T., Dorris, M., Ji, D. D., and Arnot, D. E. (1999). Analysis of *Plasmodium falciparum* PfEMP-1/*var* genes suggests that recombination rearranges constrained sequences. Mol Biochem Parasitol *102*, 167-77.

Warrell, D. A. (1992). Cerebral malaria. Schweiz Med Wochenschr *122*, 879-86.

Warrell, D. A. (1997). Cerebral malaria: clinical features, pathophysiology and treatment. Ann Trop Med Parasitol *91*, 875-84.

Warrell, D. A. (1987). Clinical management of severe falciparum malaria. Acta Leiden *55*, 99-113.

Warrell, D. A. (1999). Management of severe malaria. Parassitologia *41*, 287-94.

Watkinson, M., and Rushton, D. I. (1983). Plasmodial pigmentation of placenta and outcome of pregnancy in West African mothers. Br Med J (Clin Res Ed) *287*, 251-4.

Weatherall, D. J. (1996). Host genetics and infectious disease. Parasitology *112*, S23-9.

Whiteley, H. E., Everitt, J. I., Kakoma, I., James, M. A., and Ristic, M. (1987). Pathologic changes associated with fatal *Plasmodium falciparum* infection in the Bolivian squirrel monkey (Saimiri sciureus boliviensis). Am J Trop Med Hyg *37*, 1-8.

Wilson, R. J., Denny, P. W., Preiser, P. R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D. J., Moore, P. W., and Williamson, D. H. (1996). Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. J Mol Biol *261*, 155-72.

Yamada, M., Steketee, R., Abramowsky, C., Kida, M., Wirima, J., Heymann, D., Rabbege, J., Breman, J., and Aikawa, M. (1989). *Plasmodium falciparum* associated placental pathology: a light and electron microscopic and immunohistologic study. Am J Trop Med Hyg *41*, 161-8.

Appendix 1

Nucleotide sequences of *var* cDNA clones

D67	ATTGTAAGAGGAAAAGATCTATATA GTGGTAATAAGAAAAAGGAAAAATT AGAACAGAATTTACNAAAAATTTTC AAAGAAATATATGACAAATTGAATG GCGCCAAAGACCACTACCAAAAGGA CGGTGATAAATTTTTTCAATTAAGG GAAGATTGGTGGACAGCGAATAGCA CCCGTGTGGAAAGCATTAACATGCA AGGCGGAAGGTGCATATTTTCGACC ACGTGCAGTGATGGAAATATC
D33	ATCGTACGCGGCAAAGATCTATATA GTGGTAATAAAAAAAAAAAGCAAA CAGAAAGAGAAAAATTAGAAAATA ATTTGAAAACAATTTTCGGGAAAAT ACATAGTGACGTGACGAAGAGCGGC AATAATAAGGATGCGCTACAAGAAC GCTACCCAAAAGATGAAAAAGACG GAAATTTCTTTCAATTACGAGAAGA TTGGTGGTATGCGAATCGCGCCACA ATCTGGGAAGCATTAACATGTAACG CTCAAGGTAATACATATTTTCGACA AACAGCGTGTGCAGGAAGAAATGCG ACTCGTAATCAATGCCGGTGTGACG GCGCAAATATTGTCCCCACATATTTT GACTATGTGCCGCAGTATCTTCCTTG GTCGAAAAATGGGCAGAAAA
D215	ATTGTCAGAGGAAGAGATCTATATC GTGGTAATAATAGAGAAAATGATAA ATTAGAAAAGAAATTAAAAGGATAT TTTAAGAAAATATATGACAATTTGG

	TTGAGAAGAAGAAGGAGGAAGCAG AAACAGACTACAAAGATGATGCTCC ACATTTTTATCAATTAAGGGAAGAT TGGTGGGCACTTAATAGACAAGATG TATGGAAAGCTATCACGTGCGACGC ACATGACTCTCGTTATAGGAAAATG GGAGCAGATGGCAGTATAACAGAAT CGGCTATGAGACAATGTAGAAACGT TGCAGATGTTCCCACTAATTTCGATT ATGTGCCGCAGTATCTTCGCTGGTTC GAGGAATGGGCAGAAAATTTTTGTC GTTACGAAAACCTTAATTTA
D110	ATAATTAAAGGAACCGATTTGTGGG ATGGAAATAAGGAAGAAACAGATA CCCAAAGAAATTTGGTAACAATATT TGGTAAAATTAAAGACAAAATTCGT GATGAAGCAACCAAAAAAAAATAT AGCGACGCTCAAAAACATTTACAAT TACGTAAGGATTGGTGGGAAGCAAA TCGAGACCAAGTTTGGAAGGCGATG CAGTGTGGCAACGACAACCCGTGTA GTGGTGTAAGTGGTGTCCCATTGGA TGATTACATCCCACAAAGATTGCGT CGGATGACTGAATGGGCCGAATGGT TTTGCAAAATGCAATCACAGGAGTA TAACCAATTGATGGAAGCTTGTACT GGGTGTTTGAAAAAGGTTAGGATGG TGAAAGATGTTCCCCAAAACCAG
C89	ATTATTCGCGGAAAAGATCTATATC TTGGAAAAAAAAAAAAAAAAACAG AAACAGAAAGAGATCAATTAGAAA GTAAGTTGAAAAAAATTTCCGGGGA TATATATAATGTGTTGACAAATGGG AGGAATGGTGTAAAAGACCACTACC AAAATGATAATGGCGGAAATTATTT

	TCAATTACAAGAAGATTGGTGGACG GCGAATCGTGCCACAGTATGGAAAG CCATCACTTGTAAGGCGGACACTGG TAATGCATATTTTCGACCAACGTGC AGTGATAGTGATGGTAAAGGAAGTT TTTCTCAAGCTAATGACAAATGCCG CTGTAAGGACAAAAAGGGCAAAAA TACCGACCAGGTCCCCACATATTTTG ACTATGTTCCGCAGTATCTTCCGCTG GTTCGAAGAATGGGCC
C53	ATTGTCAGAGGAAAAGATCTTTTTA TAGGTTATAATCAAAAAGATCGAAA AGAAAAAGAACAATTACAAAATAA ATTGAAATATATTTTCAAGAAAATA CATGAAAAATTGGATTCAGAAGCAC AAACTCGCTACAATGATGCAACTGG AAATTTTTATCAATTAAGAGAAGAT TGGTGGACTGCGAATCGCGCCACAA TCTGGGAAGCCATGACATGTAGCGA GGACCTAAAAAATTCTTCATATTTTC GACAAACATGTAGTGACGAACGAAG TGGAGCCCAAGCTAATGACAAATGC CGATGTCCCAATGGTAACAACCAAG TCCCCACATATTTTGATTATGTGCCG CAGTATCTTCGCTGGTTCCAAGAAT GGGCCGAAAA
C43	ATTGTAAGAGGAAAAGATCTGTATC CCGGTTATGACGATGAGGAAAAAAA ACAAAGAGATGAATTAGAAGGAAA TTTGAAAACAATTTTCAAGAATATA TATGGCAATTTGGATAAAAAAGATC GCTACGAAGGTGATACTAAAAATTA TTATCAATTAAGAGAAGATTGGTGG TATGCTAATAGAAGACAGGTATGGA AAGCTATCACGTGCGACGCTAAGGC

	TTTTAACTATTTTAGAAATACATGTA ATGGAGAAAGTCCAACTAAAGGTTA CTGCCGGTGTAACGACGACCAGCCA AATGCCGACAAGCCAAATACCGATC CCCCAACCTATTTTGACTATGTGCCG CAGTATCTTCGCCGGTTCGAGGAAT GGGCAGAAAGACTTTTG
C37	ATTGTGAGAGGAAGAGATCTATTTC GTGGTAATGATGAAGAAAAAAAAA AAAGAGATGAATTAGAAAAGAATTT GAAAACAATTTTCGGGAAAATACAT AGTCGATTGACGAAAGACGCACAAA ATTACTACGAAGATAATGATACTGA CAAAAACTATTATCAATTAAGAGAA GATTGGTGGAAGGTCAACAGAGATC AAGTATGGGAAGCCATAACATGTGA AGCAAAAAGCGATGATAAATATAAT GTAATAGGTCCAGATGGCAAAATAA CAGAATCTAATAAGGGACAATGTGG ATGCTTTAGTGGAGATCCTCCTACTA ATATGGACTATGTGCCCCAGTTTCTT CGCTGGTTCGAGGAATGGGCCGAA
C208	ATTATACGAGGAAAAGATCTGTATC TTGGATATAATAGAAAAGAGAAAGC ACAAAAAGAAAAATTAGAACAAAA TTTAAAAAAATTTTTCCAGAATATA GATGAGAAATTGCCTCTAAAAGCAA AAAATTACTACACAAAAGAAAAAG ATCCAAATTTTTTAAAATTACGAGA AGATTGGTGGACGGCGAATAGAGAA ACGGTATGGGAAGCATTAACATGTG ATGCGCACGGTACATATTTTCATGC AACGTGCAGTGATCTTAATGGAGAT TGTTCTCAAGCTCATGAAAAATGCA GGTGTCCCAAGACAAGTGGCGGAAA

	GGCCATCAAGGCAGGTGGCGACGTA ACTATTGTCCCTACGTATTTTGACTA TGTGCCGCAAATATCTTC
B16	GTTTTGCGGATATAGGAGATATTATT CGTGGAAAAGATTTGTATCGTCGAG ATAAAGGAGAAAAAAAAAAAATAG AAGAACATTTAAAAACAATTTTCGG GAAAATACATAGTGACGTGACGTCT AGCGGGAGTAATAAGGAGGCGCTAC AAGAACGCTACAATGGTGATAAAGA AAATTATTATAAATTACGAGAAGAT TGGTGGACTGCAAATCGAGAAACGG TGTGGGAAGCAATTACATGTGACGA CGACGACAAGCTAGCAAATGCTTCA TATTTTCGTGCAACGTGCAGTGATA GTGATGGTAAAGGAAGTTTTTCTCA TGCTAATGACAAATGCCGCTGTC
A77	ATTATTAGAGGAAAGGATATGTTTA AACGTAATAAACATGATAATATAGA AAAGGGTCTAAGAGAAGTTTTTAAG AAAATATACGAGGGTTTAAAGAATA ACGGGGCGAGAGAGCATTACAAAG AAGTCAAAAATGGAAATTACATAAA ATTAAGGGAAGATTGGTGGACAGCG AACAGAGATCAAGTATGGAAAGCTA TAACGTGTGAAGCGCCAGAAAATGC ATATATTATAAAAAGAAGAATTGAT GGTGGTGATATTGAGAATTTGATTTT GACACATCCAAAGTGTGGACATGAT ACTGATCCCCCTGTTGTTGATTATAT CCCTCAACGCTTAAGATGGATGAGC GAATGGTCTGAATATTTCTGTAATGT TATTAAATA
A71	ATTGTCAGAGGAAAAGATCTTTTCC TTGGTCATAAACAAAGAAAAAAAGA

	ATTAGAAAAAAGATTAGTAGAAATG TTTAAGAACATTCAGGGTAATAATT CAAAACTCCGAACTCTTTCACTAAA TCACGTAAGAGAATATTGGTGGGCA CTTAATAGAGACCAAGTATGGAAAG CTATCACGTGCGACGCGGACACTGG TAATGCATATTTTCGAACAACGTGC AGTGATAGTCATCGTAGTGGAACTT TTTCTCAAGCTAAGGATAAATGCCG CTGTAAGGACGAAAACGGCAAAAAT GAGACCGACCAGGTTCCCACATATT TTGACTACGTGCCACAGTTTCTTCGC TGGTTCGAGGAATGGGCAGAAAGAC
A59	ATTATCAGAGGAAAAGATCTTTTCA TTGGTTATGATAAAAAAGATAGAGT ACAAAAAAAAAAATTACAAGATAGT TTGAAAAACATTTTCGGGAATATAT ATAATGAGTTGACCACGAGCGGGAA GAATGTGGACAAAGCAAAAGCTCGC TACAATGATCCTAAAGGAGATTTTC TTCAATTACGAGAAGATTGGTGGGC ACTTAATAGAGAAAAAGTATGGAGT GCTAACACATGCAACGCTCAAGGTA ATAAATATTTTCGACCAACATGTTCT GGTGGAGAAAGTATCGCTCATAATA AATGCACATGTTATTAATGGAGATC CTCCTACG
A25	ATTATACGAGGAAAAGATCTGTATC TCGGTGATATAAAAAAAAAGCAAAA TGGAAAAAAAACAGAAAGAGAAAA ATTAGAAGAGAATTTAAAAAGAATT TTCGGGAAAATACATGAGGACGTGA CGAATGGGAAGAAGGAGGCGCTAA AAACACGCTACAAAGATATTAATGA TCCAGAATTTTTTAAATTACGAGAA

	GATTGGTGGACTGCGAATCGCGCCA CAGTATGGAAAGCCATCACATGTCA TGCAGGAGAAAGTGATAAATATTTT CGAAACACATGCAATGATAGTGAAC ATAGTGGAACTTTTTCTCCACCTAAT AAATACTGCCGGTGTTACGGCGACA AACCAGTTGAAGACAAGGCAAATGT CGATCCCCCCACCTATTTTGAT
A58	ATTATTCGCGGAAAAGATCTATATC GTGGTAATAACAAAGAAAAAGATA GATTAGAAGATAATTTAAAAAAAAT TTTCAAGCAATTATATGAAGAATTA ACAAAGAACAATAAGAATGAGGCG ATAAAAACTCACTACCAAGATGATG ATCCAAATTATTATAAATTAAGGAA TGCTTGGTGGGAAGCTAATAGACAA GAAGTATGGAAAGCTATCACGTGCG GTGCTGGGGGTTCTAAATATTTTCGA CACACATGTGGTACAGGAACGCCGA CTGATGATAAATGCCGATGTTG
A51	ATTATCAGAGGAAAAGATCTGTATG AGGGTTATGATCAAAAAGATAAAGA ACAAAAAGTTAAATTAGAAAATAAG TTGAAAGATATTTTCAAGAATATAT ATAATGAGTTGACGTCGACGAATGG GAAGAAGGGGAAGAAGCAGGCGCT ACAAGCTCGCTACCAAGATGATGGA TCTGGAAATTATTATCAATTACGAG AAGATTGGTGGGATGCGAATCGCGC CAAAGTATGGTATGCTATCACTTGT GGTGCAGGAACTAGTGATAAATATT TTCGAAAAACATGTTCTAATGACAC ATCCGACACTAACGAAAAATGTCGA TGTGTCCGTACCGATCCTCCTACGTA TTTTGACTACGTGCCGCAGTATCTTC

	CCTGGTTCGAAGAATGGACC
A50	TTGTAAGAAGAAAGGATATGTTTAA ATCTAATGACAATGTAGAAAACGGT CTAAGAGAAGTTTTTAAGAAAATAT ACGAGGGTTTCTTGGATAAGGGGGC GAGAGAGCATTACAAAGAAGTCAA AAATGGAAATTACATAAAATTAAGG GAAGATTGGTGGACAGCGAACAGA GATCAAGTATGGAAAGCCATGACTT GTGTTGCACCAGAAAATGCTTATTTT AGAAAAACAGAAGCTGATGGAATA GGAATTTCAAGTTTAATTTTACCATA TTCTAAATGTGGACGTGATACTGAT CCCCCTGTTGTTGATTATATCCCTCA ACGCTTAAGATGGATGAGCGAATGG TCTGAATATTTCTGTAATGTATTAAA TAAAGAAATAGATGAAATGA
A46	ATTGTCAGAGGAAGAGATTTGTATC GTGGCAATAGAAAGAAAAATGAAA CAAAAACAGAAAGAGAAAAATTAG ATGATAAGTTGAAAGAAATTTTCGA AAACATTAGGAAAGAGAATAATTCA AAACTTAAATCCCTTACAGATGACC AAATTAGGGAATATTGGTGGACTGC TAATAGAAACGATGTATGGAAAGCT ATCACATGTAACGCGGACAAGGGTA ATACATATTTTCGAGCAACGTGCATT GATCTTAATGAAAGTCCATCTATGG CTAGAGACAAATGCCGCTGTAAGGA CAAAAGCTTCACAAAAGAGACCGAC CAAGTCCCCACATATTTCGACTACGT TCCGCAGTTTCTTCCGCTGGTTCGAA GAATGGGCAAAAAATTTTGT
A38	ATTATAAGAGGAAAAGATCTATATC TTGGAAAAAAAAAAAAAAAGCAAA

	ATGGAAAAGAAACAGAAAGAGAAA AATTAGAAGGTAATTTGAGAAAAAT TTTCGAAAACATTAAGAAAGAGAAT AATTCAAAACTTAAATCCCTTACAG GTGACCAAATTAGGGAATATTGGTG GACAGAGAATCGTGAAACAGTATGG AAAGCCATGACTTGTTGCGAGGACC TAAAAAATTCTTCATATTTTCATGCA ACGTGCATTGATGGAAAGAATCAGT CTCAAGCTAGAAACCAATGCCGATG TCCCAAGACAAGTGGCAACGTAAAT ATTGTTCCCCCATATTTTGATTATGT GCCACAGTATCTTCGCTGGTTCGAG GAATGGGCAGAAGACTTTGTC
A211	ATTATTCGTGGAAAAGATTTGTATC GTCGAGATAAAGGAGAAAAAAAAA AATTAGAAGAACATTTAAAAACAAT TTTCGGGAAAATACATAGTGACGTG ACGTCTAGCGGGAGTAATAAGGAGG CGCTACAAGAACGCTACAATGGTGA TAAGGAAAATTATTATAAATTACGA GAAGATTGGTGGACTGCTAATCGAG AAACAGTATGGAAAGCTATAAGGTG TAGTGCGCCAAGGGATGCTGATTAT TTTATAAAAACAGTGTGTTCTGGAG GAAAAACTCCGACTCAAGGTAAATG CCGATGTATCGATTTTAGTGTTCCCA CATATTTTGACTACGTGCCGCAGTAT CTTCGGTGGTTCGAAGAATGGGCCG AAGACTTTTG
A21	ATTGTACGCGGCAAAGATCTATTTC GTGGTTATGATGATGAAGAAAAAAA TCGAAGAAAAAAATTAGAACAGAA ATTGAAAGTTATTTTCGGGCATATAT ATGAAGAATTGAAGAAGCACAAAA

	AGCTTAAGGAGGAAGCAGAAGAAC GCTACAAAAAAGACGGTGATAATTA TTATAAATTACGAGAAGATTGGTGG GCACTTAATAGACAAGAAATATGGA AAGCTATCACGTGCGGACATCCGGG TGGTACATATTTTCGACAAACAGCG TGTGGTGGAGGAACAACTCCGACTC CTAATAAATGCCGATGTGCGACAAA TGATGTACCTACATATTTCGATTATG TGCCGCAGTATCTTCGCTGGTTCGAA GAATGGGCAGAAGATTTTTGT
A185	ATCGTACGCGGCAAAGATCTGTATC TTGGTAATAATAAAGAAAAAAAGCA ATTAGAAGAGAATTTAAAAAAGATT TTCAAAGAAATATATGACAAATTGG ATGGGAAGAACGGGAAGAAGACGC TACAAGAACGCTACAAAGGTGATAC TACAAATTATTATCAATTACGAGAA GATTGGTGGAATATCAATAGAAAAA AAGTATGGGATGCTATCACATGCGG CGCTGCGGGTGGTACATATTTTAGA AAAAGACGTGTTCTAGTTATTATCC AACTGGAGAAGACTGCCGATGTGTG GCAAATGTTCCCACATATTTCGATTA TGTGCCGCAGTATCTTCCCT
A177	CATTATACGAGGAAAAGATCTGTAT CGTGGTAATAATGGAAAGGATAAAT TAGAAGAGAATTTGAAAACAATTTT CGGGAAAATACATGAAGGATTGAAG AATGGGAAGACGAATGGCATAGAA GAACGCTACGGAAATGATCCAGATT TTTTTCAATCAAGAGAAGATTGGTG GACTGCTAATCGAGAAACAGTATGG AAAGCTATAAGGTGTAGTGCGCCAA GGGATGCTGATTATTTTATAAAAAC

	AGTGTGTTCTGGAGGAAAAACTCCG ACTCAAGGTAAATGCCGATGTATCG ATTTTAGTGTTCCCACATATTTTGAC TACGTGCCGCAGTATCTTCGGTGGTT CGAGGAATGGGCCGAAGACTTTTGG
A169	ATCGTTAGAGGAAGAGATCTATATA GAGGTAATAAGAAAGAAAACAAAC AAAGAGAAAAATTAGAAGAAAATTT GAGAAAAATTTTCGAGAATATATAT GAAGGATTGTCGAACAATGGTGTAA AAGCTCGCTACGAAGGTGATAAAGA AAATTTTTATCAATTACGAGAAGAT TGGTGGGCTCTTAATAGAGACCAAG TATGGAAAGCATTAACATGTAGCGA CGACCTAAAAGATGCTTCATATTTTC GACCAACGTGCAGTGATCGTAAAGG AATTGTTCTCAAGCTAAGGATAACT GCCGGTGTGACGGCTCAAATACCGA CCAGGTCCCCACATATTTTGACTATG TGCCACAGTTTCTTCGCTGGTTCGAA GAATGGGCAGAAGACTTTT
A150	ATTGTCAGAGGAAAAGATCTTTTCC TTGGTCATAATCAAAGAAAAAAAAA ATTAGAAGAAAGATTGGAACAAATG TTTAAGAACATTACGCATAGTAATG CGGCTAAACTAAGTGTACTTTCAAG CAAAGAAATTAGGGAATATTGGTGG GCTCTTAATAGAGACCAAGTATGGA AAGCACTAACATGTGACGAAGAGAA CAAGCTAGGGGGTAATGCATATTCT CATGCAACGTGCAGTGAACGTAATG GAGGTTGTTCTCAAGCTCATGAAAA ATGCAGGTGTCCCATGACAAGTGAT GGCAAGCCCAATGACCAAGTCCCCA CGTATTTTGACTACGTGCCGCAGTAT

	CTTCGCTGGTTCGAAGAATGGGCAG AATATTTTTGCAAGAAAAAA
A149	CGTTAGAGGAAAAGATCTATTTTAT GGTAATACATATGAAAGTACACAAA GAAAAGTATTAGACGATAATTTAAA AACAATTTTCGAAAACATTAAGAAA AGTGATACAAAACTAACTAAGCTTA ATGATGAACAAATTAGGGAATATTG GTGGGAAGCCAATCGAGAAACAGTA TGGAAAGCCATAACATGTAGCGACG ACCTAAAAAATTCTTCATATTTTCGA ACAACAGCGTGTGCAGGAACGCGGA CTAATGACAAATGCCGCTGTACCAA GTCAAGTGGCGCTAAGGTCG
A145	ATAATTAGAGGAAAGGATCTTTGGA ATACAGATAAAAATTATAAGAACAT ACAAAACAAAATAAGATACGTTTTC GATTATATGCATAAGAAGTTGAATT CTGACGATCAAAAGAGATATAAAGA TTTAGTGAATCATTATGATCTACGTC CTGATTGGTGGGATGCTAATAGAAG AGACATATGGAAAGCTATGACTTGT GCTGCACCAAGAAATGCTTATATTC ATAAAACAACAGAAAATAGTGAAA CAAAAATTCGTTCAACAGATATGTA TTATTATTGTGGACATAAGGATGAT CCACCTGATAATGATTACATTCCAC AAAAACTAAGATGGATGACTGAATG GTCCGAATACTTTTGT

Appendix 2

Nucleotide Sequence of *var*-CHO 3 clone

5'-CGAAGTTTTGCGGACATAGGAGATATAATTAAAGGAACAGATTTATGGGA
TAAGAATGGTGGCGAACAAAAAACACAAGGAAAGTTGGAAAAAATATTCTG
TAAAATTAAAAACAAACTTCCTGAAGATATCCAAGAAAAATATATCAATGATG
ACAAAAATTCACCCCAATATAAAAAATTACGAGAAGATTGGTGGGAGGCAA
ACAGAAAAGAAGTTTGGAGAGCTATGACGTGTGCAACAACCAGTGGAAAAA
TCCCATGCAGTATTGTCACACCACTTGATGATTACATTCCTCAAAGATTACG
TTGGATGACTGAATGGTCCGAATGGTTTTGCAAAGAGCAGTCGAAACTGTA
TGGAGAGTTGGTGAAGGATTGTGCAAGTTGTAAGAAAAAGGGTAAAGAAAA
ATGTACACAAGGCGATAATGACTGTACACCGTGTGATAAAAAATGTAAAGA
ATATGGAAAAAAAATAAGAACATGGAAAGATCAATGGACAAAAATGGATGG
AATATACCAAATGTTATACCTACAAGCACAAACTACTGCTCGTAATGCTGGT
GATACTGCTTTTGATAATCCCAACGATCAATATGTGATTGATTTCTTCAAAAA
ATTACAAAAAGCAAATGGTGATAACAATTTTGGTGTCAACACCTCCCCGTAC
TTCACCCCAGCAGGGTACATCCACCAGGAGGCACGTGTAGGTGAATGCGA
AGTACAAAAACATTTTTGTAATAATAACGGCAACCAGGATAAATATTCCTTTC
GTAATCAACCATATGACCACGAAGAAGCATGTGCTTGTAAAAAGAATACTAA
AGCACCAGAAAAGAAGAAAGAAGAAACACCACCAGCGGGACCAAACGTTT
GCGAAATAGTGGACAAACTATTTGAGGACACCACTACACCTCACGCAGCTT
GCCAACAAAAGTATATAAATGGCCACGAAAAATTCCCCAATTGGAAATGTGT
CACACCAAGTGGTGAAAAAAGTGGTGATAAGGGTGCCATATGTGTCCCACC
CAGGAGRCGA-3'

Nucleotide Sequence of *var*-CHO 3' clone

5'-CCGAAAAAACTATATTTCCCAACTCCCGCACTACAAAATGCGATGTTATC
CAATACCATTATGTGGACTATTGGAATTGGTTTTGCTGCAATCAGTTACTTT
TTTCTAAAGGTATTATATATGTATATATATGTGTATGTATGTGGATGTGGAT
GTGGATGTGGATGTATATGTGTTTTGTGGATATGTATGTGTGGGTGTTTTG
GGATATATATATATGTGTTTCGTGTATATGTATGTGAAGTATTTAGATATATA
TATTTGTATTTATATGTATGTGTGAGTATTTGGATATATATATGTTTCTGTAT
ATGTGTTTTGTGTAAATGTATTTATTGGTGTTTGGATGTATATATATATCTGT
TGATGTATATGTGTTCCTGTATATTTATTTGTGGGTGTTTTTGTATATATATA
TATGTTGATGTATATGTGTTTTGTGTATATATATGTGTGTTTGGATATATATA
TATGTGTTTCTGTATATGTAGGTGTGGATATGTTT-3'

Appendix 3

Nucleotide sequence of *Var*-CHO (exon 1)

ATGAAAACAATATGTATGTATATGGATGAGTGTATTTACGACATAATGTAGG
CCTGAACAAAAAAAAAAAAAAAAAAAAAAAAAATGGGTCCCGCGCCTAGG
ACAGCAACCACTACATACAGTAGTGCCAAGGACCTTTTGGAAGACATTGGA
GAAAGTGTGCAGAAAGAAGCAAAAAAGCAAGCTCTTGGACGTAGTGAGAGT
GTTTTGCATGGCTTGTTGTCAAATGCAACAATTAAAGGTGTGAAAAACAAAG
CCACAAAACCAATCCAACTTGAATATGAATATCACACTAACGTTACCGGTGG
TTTTGATAAAAATAATCCTTGCGCAAATAGGTTGGACGTCCGATTTTCCGAT
ATATATGGAGGTCAATGTACTGATAATAAAATAAATGGAAATGATGATGAA
ACTGGTGGAACGTGTGCTCCATTAAGGAGGTTATTTCTATGTGATCAGCATT
TGTCGCACATGAAGGAAGGTAATATTAATAACACAGATAATTTATTATTAGA
GGTGTCTCTTGCAGCAAAATATGAAGGAGATTCAATAATTAATAACTATCCT
GATAACCGTGACAAAAAAGAAGGAATTTGTACAGCATTGGCACGAAGTTTT
GCTGATATAGGTGACATTATCAGAGGAAAAGATCTGTTCTTGGTTATACCAA
AAAAGATTGAAAAAGAAAAAGAAAAAGTACAGAAAAATTTAAAAAGAATA
TTCAATGAAATATATAAAAAAATGCAAGATCCTGCAAAATCTCATTATAGTG
GTGATTCTTCAGATTTTTATAAACTAAGAGAAGATTGGTGGGCACTTAATAG
AAAAGAGGTATGGAAAGCTATCACATGTAAAGCAAAAAATGATGCTGAATA
TTTTAGAAAAAAAAGATTCTGATGGAAAACATTGCTCTGTTCAAAATTGCAAA
TGCGTTGATGGAGATCCTCCTACAAATCTAGACTACGTTCCTCAACATTTAC
GTTGGTTTGATGAATGGAGCGAAGAATTTTGTAGAAAAAGAAAAGATCAAT
TAAAAATTTCTTTAGAGAAATGTCGTGGAAAAAATAAAGATAAATATTGTA
GTCTTAATGGATGCAACTGTAAAACAACTGTTAGAGGAAAAAAAAAATTCG
ATTATCAACAAGAATGTAATGACTGTTTGGTTGCATGTGATCCTTTCGTACAT
TGGATAGATAACGAAAAACTAGAATTTTTAAAACAAAAAAAAAAAATATAAA
AATGAAATAAAAGAAAGAGAACCTACAAAAAAAACATCACATGGAACTATC
AATAATATGTATGCCAAAGAGTTTTATGAAACACTGGAAAAGGAACATAGA
ACTGTCGATACATTTTTAAAATTATTAAATGAAGAAAAAGAATGTAAAAATC
ACCCTGAAGTAGGAGAGGGAAAAAAAAACTTTTATTGACTTTAATGATAATAT
CGAAACATTTTCTCATACAAAAATTTGCGAACCTTGCCCTTGGTGTGGAGTG
AAACCGGGCGGTCCACCATGGAAAGATAACGACATTGATTCATGCGGGAAA
AAAGAAATATCGTTTAGCGATAAAGATACTACTGATATATCAATACTTTCCA
CTGATAGAGCAAAAAAAAAATATTTTACAAAAATTAGAAAATTTTTGCAGAG

TTCACCCCAGCAGGGTACATCCACCAGGAGGCACGTGTAGGTGAATGCGAA
GTACAAAAACATTTTTGTAATAATAACGGCAACCAGGATAAATATTCCTTTC
GTAATCAACCATATGACCACGAAGAAGCATGTGCTTGTAAAAAGAATACTA
AAGCACCAGAAAAGAAGAAAGAAGAAACACCACCAGCGGGACCAAACGTT
TGCGAAATAGTGGACAAACTATTTGAGGACACCACTACACCTCACGCAGCTT
GCCAACAAAAGTATATAAATGGCCACGAAAAATTCCCCAATTGGAAATGTG
TCACACCAAGTGGTGAAAAAAGTGGTGATAAGGGTGCCATATGTGTCCCAC
CCAGGAGNCGACGACTATACATACACGATTTACAATCACTAGACGAGAAGC
CGAGCGATACCGCGCTACGTGATTGGTTTGTGAAAAGTGCCGCGGTTGAGAC
GTTTTTCCTATGGCATAGATACAAAAAACAAAAGGAAAAGAAACCACAGGA
AGGGTCACTACTTTCCGGGTCAACACTACTTAGCCAGTTCAGCATAGACCTC
GGTGATGAGGAACAGCCCCCCGAAAAACAATTGGCTGGTGGCAAAATACCT
GATGATTTCTTGCGCCAAATGTTCTACACATTGGGAGACTATAGAGATATAT
GCATAGGTGATGAAAACGTGATTAAGACGTTAAAATATAGTGGTGATAAAG
ACGACATTATGGAGAAAATACAAGAGAAAATAAAGGCTGTTTTTCCAACTA
GTGGCGGCACATCTCACATACCTGATGTCAAACCTGGTGGCGCCCTATCGCG
CGGCGACTGGTGGGAACAACATGGTAAAGATATATGGCATGGAATGATTTG
CGCTTTAACCTATAAAGAGACAAGTGGTAGTGGCGAAAAGGGTGAAAAAAC
AACTATTACCCAGGATGGTACTCTGAAAGATGCACTTTTGGATACCGACGGC
AAAAAGCCAAAACGTGACTACCAATACACAAGTGTCACACTTAAAGAAGAT
GATAGTGGAGAAAAAGCCGCCAATGTCCCGGCCAAAGTGGAACCCACTACA
CTGGCCGACTTCACGCGCCGCCCAGCGTACTTCCGCTGGCTGGAAGAGTGGG
GCGACGGCTTCTGTCGAGAGAGGAAAAAACGATTGGCACAAATTAAAAAAG
ATTGCTACGAAGACGGTGGAACAGGTGAAAAACAATACAGCGGTTATGGGG
AAGCGTGTGATCGAACAAATACATCAAATGAAGGAGCTTCTGCTGATTTAG
AAGGTCCAAGTTGTGCAAATTCTTGTAGTTCTTATAGAAAATGGATAGAAAG
AAAAAAATACGAATTTAAAAAACAAGAAAAGGCATATGGTGGACAAAAAC
AAAACTGCAAAAAGGAACGTAAAGCTGCTGAAAGTAATGATAATGATAAAC
AATTTTGCGGAACACCAGAAACAACATGCAATACAGCTGAAGCATTTTTACA
AAATTTAGGATCATGTAAAAAGTATAATGGAGAGGGTAAAAAAATTTTTGA
GAATACAGAGGAAACATTTAAACCTGCAATCGATTGCGAACCATGTTCTGA
ATTTAAAGTTAAATTAGAAAAGTGTAATTGCGGGAGTGATGCTAAAGGAAA
TACTTGCACAACAGGAAAGATTACGGCAGAAAATTTTGAAAATAAGACAGA
TGTTAATGAAGTTGTTATGCGTGTTAGTGATAACGCTGAAAGTGGTTTTAAA
GGTGATTTAAAGAGTTCTTGTGAAAACGCACATATTTTTGAAGGTATTAAAG
AAAATAAATGGAAATGTCGTAATGTATGTGGTTATATTGTATGTAAACTGGA

AGAAGTCAATGGGGAAAAAGATAATGGTAAAAAAATTTTATTAATTAGAGC
ATTGGTTACACATTGGGTAGATAATTTTTTACAAGATTATAATAAAATTAAA
AAAAAATTAAACACATGTATGAATAGTAGTGATGCAACCCCATGTATAAAG
GGATGCGTAGATAAATGGATAAAGCTGAAAAAGGATGAATGGGAAGAAAT
AAAAAAACCTTACCTTGAACAATATAAAAATGGCTATGGAGAAAATTACAA
TGTGAAAACTATTTTGGAGAAATTTCAAGATCAACCTGAATTTAAAAAAGCT
ATAGGACCTTGTCCAACTTTAGATGCTTTCGAGAAGTCAAAACAATGTAATG
CAACTGCGAGCTCAGAAAAGGGTAAAGATGGTAACAAAAGTTATGTTATAG
ATTGTTTGCTTCAAGAGCTTGAAAAGCTTCAAGAAAAAGCAAAAAAGTGTC
ATGACCAACATAGCGACAATCCACAAGAAAAATGTGATGATCCCCACCCTG
ACGAACCCGATGAGGAGGACTTACTCCTTGAAGAAGAAGAAAATACGGCAA
ATAGTGCGCCAGAAATTTGTAAAGATGTGATAAAAGCACCACCAAAACAAG
AGGAAAAAGGCGGTTGTGAACCAGCATCCCCTCTTGAACCCGAAGAAGTGG
AAGAAGAAACAGCTAGTGTCCCACCTGGATCAGAACCTGAAGCTGATAAAG
GCCCAGTCAAACCCGCAGAACTACCAAAACCACCAAAACGAAACAAAAGGC
AACCGAAAAAACTATATTTCCCAACTCCCGCACTACAAAATGCGATGTTATC
CAATACCATTATGTGGACTATTGGAATTGGTTTTGCTGCAATCAGTTACTTTT
TTCTAAAGGTATTATATATGTATATATATGTGTATGTATGTGGATGTGGATGT
GGATGTGGATGTATATGTGTTTTGTGGATATGTATGTGTGGGTGTTTTGGGAT
ATATATATATGTGTTTCGTGTATATGTATGTGAAGTATTTAGATATATATATT
TGTATTTATATGTATGTGTGAGTATTTGGATATATATATGTTTCTGTATATGT
GTTTTGTGTAAATGTATTTATTGGTGTTTGGATGTATATATATATCTGTTGAT
GTATATGTGTTCCTGTATATTTATTTGTGGGTGTTTTTGTATATATATATATGT
TGATGTATATGTGTTTTGTGTATATATATGTGTGTTTGGATATATATATATGT
GTTTCTGTATATGTAGGTGTGGATATGTTT

Molecular and Biochemical Parasitology 102 (1999) 167–177

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

www.elsevier.com/locate/parasitology

Analysis of *Plasmodium falciparum* PfEMP-1/*var* genes suggests that recombination rearranges constrained sequences☆

Christopher P. Ward, George T. Clottey, Mark Dorris, Dar-Der Ji, David E. Arnot *

Institute of Cell, Animal and Population Biology, Division of Biology, University of Edinburgh, West Mains Road, Edinburgh, Scotland EH 9 3JT, UK

Received 2 February 1999; received in revised form 4 May 1999; accepted 18 May 1999

Abstract

The *var* genes of *Plasmodium falciparum* encode a family of parasite erythrocyte surface antigens, the PfEMP-1 proteins, which function as adhesion ligands for host endothelial and erythrocyte receptors. PfEMP-1 is extremely polymorphic although the extent of this variation in naturally transmitted parasite populations is unclear. We have identified 56 different sequences from the Duffy binding-like (DBL-1) domain of *var* genes amplified from six different *P. falciparum* clones isolated from patient infections in a Sudanese village in October–November 1989. These clones have been compared with 25 PfEMP-1 sequences expressed from different *var* gene loci by the 3D7A clone and 48 PfEMP-1 sequences from different isolates in endemic areas such as Kenya, Brazil, Gambia, Vietnam and Vanuatu to analyse diversity in clonal, local and 'global' *P. falciparum* populations. Evidence that certain conserved sequences recur in clones from one Sudanese village and in isolates from all over the world suggests that *var* gene diversity is the result of recombinational reshuffling of a subset of conserved, presumably ancestral sequences. Recurrence of particular *var* sequence blocks thus leads to 'overlaps' in the PfEMP-1 sequence repertoire of different *P. falciparum* clones.

Keywords: Plasmodium falciparum; Antigenic variation; var gene recombination; Global diversity

1. Introduction

Plasmodium falciparum parasites are genetically diverse [1–4] and patient infections are frequently mixtures of several haploid parasite clones [5,6]. Many immunogenic *P. falciparum* antigens are polymorphic and most parasite isolates have different combinations of variants of these antigens.

Abbreviations: CIDR, cysteine-rich interdomain region; DBL, Duffy antigen binding-like region.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the EMBL, Genebank™ and DDJB data bases under the accession numbers AF127272–AF127330 and AF152572–AF152582.

* Corresponding author.

0166-6851/99/$ - see front matter

PII: S0166-6851(99)00106-1

This between-clone antigenic diversity in the parasite population is generally considered to benefit the parasite in that it renders host immunity relatively clone-specific.

The discovery of the *var* multi-gene family established that *P. falciparum* also has within-clone antigenic diversity generating mechanisms [7,8]. Each of several dozen genomic *var* genes encodes a PfEMP-1 which acts as an endothelial and erythrocytic adhesion molecule on the surface of parasite infected red blood cells. Although PfEMP-1 sequences are highly polymorphic, all *var* genes encode proteins containing 1–5 Duffy binding-like (DBL) domains with a cysteine rich interdomain region (CIDR) adjacent to the N-terminal DBL-1 domain. Switches in PfEMP-1 expression occur within clones[9] and correlate with changes in the cytoadherent and antigenic properties of the infected red blood cell [10]. This allows clonal infections to evade immune responses against essential, but vulnerable, erythrocte membrane proteins by switching between antigenically distinct but functionally similar proteins.

Two studies have shown a positive correlation between the capacity of individuals' serum to agglutinate a larger number of parasite isolates and protection from clinical malaria attacks [11,12]. The possible relationship between anti-PfEMP-1 antibody responses and clinical protection has led to the prediction that immune selection will shape the genetic structure of *P. falciparum* populations in order to minimise overlap in *var* gene repertoires. This would generate a population 'strain structure' in which each 'strain' has a distinct, non-overlapping repertoire of PfEMP-1 variants [13]. We have analysed the first 'Duffy binding-like' domain (DBL-1) of PfEMP-1 genes in *P. falciparum* clones isolated from malaria patients in a Sudanese village during the malaria transmission season of 1989. These were compared to the expressed *var* sequences of the 3D7A clone and the existing databank of PfEMP-1 sequences to analyse the *var* repertoires of 'global' and 'local' populations of *P. falciparum*. Phylogenetic analysis has been used to determine the most statistically likely relationships between these sequences.

2. Materials and methods

2.1. Parasite clones

The parasite clones SD101. SD102, SD105, SD106, SD126 and SD128 were made from six unrelated Sudanese patient samples isolated during October and November 1989 [14]. Each of these parasites has a distinct phenotype, genotype and molecular karyotype [2,14,15]. The 3D7A clone [16] derives from the Nijmegan NF54 isolate originally isolated from a young Dutch girl who spent one night on a boat several miles from Amsterdam Airport in July 1979. This isolate is thus of mysterious provenance although its sensitivity to all anti-malarial drugs makes an African origin seem most likely.

2.2. Preparation of parasite DNA and RNA

Parasites were grown to a 5% parasitaemia in culture and parasite pellets obtained by saponin lysis. Parasite pellets were digested with proteinase K in the presence of 10% sodium dodecyl sulphate, and DNA isolated by phenol chloroform extraction and subsequent ethanol precipitation [17]. RNA was extracted using the Genosys RNA Isolator kit.

2.3. PCR and RT PCR amplification of var DBL1 sequences

PCR amplification of *var* DBL1 sequences was obtained using the 5′ oligonucleotides Forward 1 (CGAGGATCCGGWGCWTGYGCWCCWTWYMG) and Forward 2 (CGAGGATCCCCATATAGACGATTACATSTATG) and the 3′ oligonucleotides Reverse 1 (GCACTCGAGTTAWATRTCYGCAAAACTKCGTG), Reverse 2 (GCACTCGAGTTANARRTAYTGWGGWACRTARTC) and Reverse 3 (GCACTCGAGTTATTCTTYTYTTTGGTTATCTATCCA). Forward 2, Reverse 1 and Reverse 3 are based on primer sequences kindly provided by Dr Sue Kyes, Oxford University [18]. Forward 1 and Reverse 2 are based on published primer sequences [8]. Reverse transcription PCR used the

Boehringer Expand Kit and cDNA primer (CCWGGWACATAWATATCATTWATRTC) followed by PCR forward primer RT1 (GCACGMAGTTTTGCRGAYATWGG) and PCR reverse primer RT2 (GTATARTCGTCGYCTCCTGGGTGGSAYAC). (Redundancies, M = A/C; R = A/G; W = A/T; S = C/G; Y = C/T; K = G/T; V = A/C/T; H = A/C/T; D = A/G/T; B = C/G/T; N = A/C/G/T).

2.4. Cloning of var DBL1 sequences into the vectors pCRII and pGEM

PCR amplified *var* gene fragments were cloned into the plasmid vectors pCRII and pGEM using the Invitrogen TA cloning kit and the Promega pGEM-T Vector System I cloning kit respectively. *E. coli* XL-1 blue was used as the host cell line for the recombinant plasmids.

2.5. Sequencing of cloned var gene fragments

The Qiagen Spin Plasmid kit was used to prepare recombinant plasmid for use as the template in sequencing reactions. Sequencing was carried out using the ABI PRISM automated sequencing system with the ABI Dye Terminator sequencing kit.

2.6. Analysis of var gene sequences

For the purposes of this analysis the first Duffy Binding-like domain (DBL-1) is defined as the amino-terminal 400 amino acids of the *var-1* type-sequence [8]. The defining features of the domain are the 16–18 conserved cysteine residues (Fig. 1). The figure shows the positions of the forward and reverse primer sequences that have been used to amplify and sub-clone DBL-1 *var* sequences for this study and the sequence blocks used to analyse phylogenetic relationships. Sequences obtained were confirmed as *var* fragments by BLAST analysis. *var* sequences from parasite isolates from the Gambia, Brazil, Indochina, Kenya, Papua New Guinea, Vanuatu, Vietnam and from 3D7A were obtained from the Genebank database. Deduced amino acid alignments of these Genebank sequences with the Sudanese and 3D7A DNA sequences were made using the GCG pileup and MacVector clustal functions. Alignments were created using program default parameters with gap weight of 3.0 and gap length weight of 0.1 and edited by hand. Phylogenetic analysis of sequence alignments from two regions of DBL-1 was carried out using the computer program PAUP 3.1.1 [19]. Further cladograms and alignments containing all of the Sudanese and 'global'

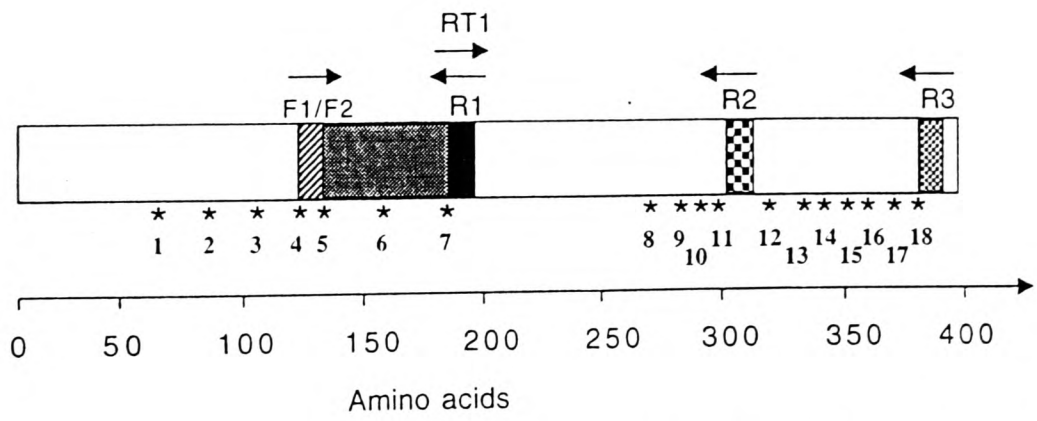

Fig. 1. A schematic model of DBL-1. The relative positions of the conserved cysteines 1–18 are indicated by asterisk. The anealing sites of primers F1, F2, R1, R2, R3 and RT1 are shown, the RTPCR reverse primer RT2 anneals to a region 3′ of DBL-1 and is not shown. The hatched area corresponds to the region of sequence aligned in Fig. 2 and used to generate the phlyogenetic model presented in Fig. 4. The black area corresponds to the highly conserved LARSFADIGDI motif and is the binding site of R1 and RT1.

Table 1
List of Sudanese *var* gene sequences amplified by different primer combinations

Primer combination[a,b]	Length of fragment (No. amino acids)	Variants[c]
F1/R1	~70	SD102A, SD102B, SD102C, SD102D, SD102E, SD102F, SD102F2, SD102G, SD102H, SD102J, SD105G, SD105L2, SD105N, SD105P, SD126E, SD126F, SD126G, SD126H, SD126J, SD126K, SD126N, SD128J
F1/R2	~200	SD105A, SD105B, SD105D, SD105E, SD105F, SD105K
F1/R3	~280	SD105C, SD105J, SD105L, SD105M, SD126M
F2/R1	~70	SD105T, SD106B, SD106D, SD106E, SD106F, SD106G, SD126D, SD128A, SD128C, SD128D, SD128E, SD128G, SD128H, SD128K, SD128L
F2/R2	~200	SD101A, SD101B
F2/R3	~280	SD105Q, SD105R, SD105S, SD105U, SD106A, SD106C, SD126A, SD126B, SD126C

[a] Primer anealing sites are indicated in Fig. 1.
[b] The sequences obtained by each primer combination are in Fig. 2. Only those sequences amplified by the primer combinations F1/R2, F1/R3, F2/R2 and F2/R3 are aligned in Fig. 3.
[c] The name of each variant indicates the parasite clone from which it originated e.g. the variant SD102A was isolated from the parasite clone SD102.

sequences analysed can be obtained from the authors.

3. Results

3.1. *var* gene sequences from clones isolated in a Sudanese village in October and November 1989

DNA isolated from parasite clones SD101, SD102, SD105, SD106, SD126 and SD128 was used to amplify the DBL-1 domains of their *var* genes. All of the sequences analysed encode at least the 50–60 amino acids between the F2 and R1 primer sequences (Fig. 1). Excluding the primer sequences, an alignment over this region of 55 different sequences from the Sudanese clones is presented in Fig. 2. Each sequence was isolated from a subclone library at least twice. Different subsets of *var* genes were obtained from each parasite clone when different primer pairs were employed. Frequently, *var* types amplified using either F1 or F2 with either R2 or R3 were also isolated when using these forward primers and R1 (R1 encodes part of the LARSFADIGI motif found in all PfEMP-1 sequences). Table 1 lists the *var* types obtained using each primer combination and the length of fragment amplified. In three cases, identical sequences were identified from different parasite clones from different patients (SD101A (F2/R2) and SD102C (F1/R1); SD102E (F1/R1) and SD126F (F1/R1); and SD126B (F1/R1) and SD128H (F1/R1)).

Fig. 3 shows alignments of 22 sequences generated using the R2 and R3 reverse primers. Figs. 2 and 3 together show Pileups aligned around 13 conserved cysteine residues (positions 6–18 in Fig. 1). Cysteine 6 is missing in 7/58 of these *var* variants (SD105A, SD106D, SD106E, SD126C, SD126D, SD126M, SD126N and SD128C). SD126D is missing cysteine 6 but has a cysteine at an unusual position (position 15, Fig. 2). Cysteine 7 is not found in 1/58 sequences (SD106E) and cysteine 9 and 11 are absent from SD105L and SD105D respectively. SD101A has an 'extra' cysteine adjacent to cysteine 8 (position 102 in Fig. 3). Cysteines 8, 10 and 12–18 are conserved in all Sudanese *var* genes examined. The large aromatic amino acids phenylalanine, tryptophan, and tyrosine are highly conserved in the *P. falciparum* PfEMP-1 DBL-1 domain, a feature also observed in the Duffy-binding antigens of *P. knowlesi* and *P. vivax* [20].

The region from cysteine 7–18 (Fig. 3) contains between 182 and 204 amino acids. In three pairs of these sequences this sub-domain contains iden-

Fig. 2. Alignment of 56 Sudanese DBL-1 sequences from six parasite clones over the region between F1/F2 and R1 (see Fig. 1). Conserved residues are boxed and shaded dark grey; conservatively substituted residues are boxed and shaded light grey. Consensus residues are shown below the alignment. The conserved cysteines at positions 28 and 61 correspond to cysteines 6 and 7, respectively. The aromatic residue tyrosine is conserved at positions 33, 42, 46 and 51. The sequences SD126B/SD128H, SD102E/SD126F and SD101A/SD102C were each isolated from two different parasite clones. Two sequences isolated from parasite clone SD105 are identical over this region, but diverge downstream.

tical numbers of amino acids. Several almost identical large sequence blocks occur in more than one PfEMP-1 protein in this region. SD105Q and SD105S are identical at nucleotide level over a region encoding 155 amino acids but diverge after cysteine 15. SD105L and SD105L2 are practically identical (53/55) residues over the region covered by the F2 and R1 primers and similarly SD102F and SD102F2 share 52/54 residues in the same region.

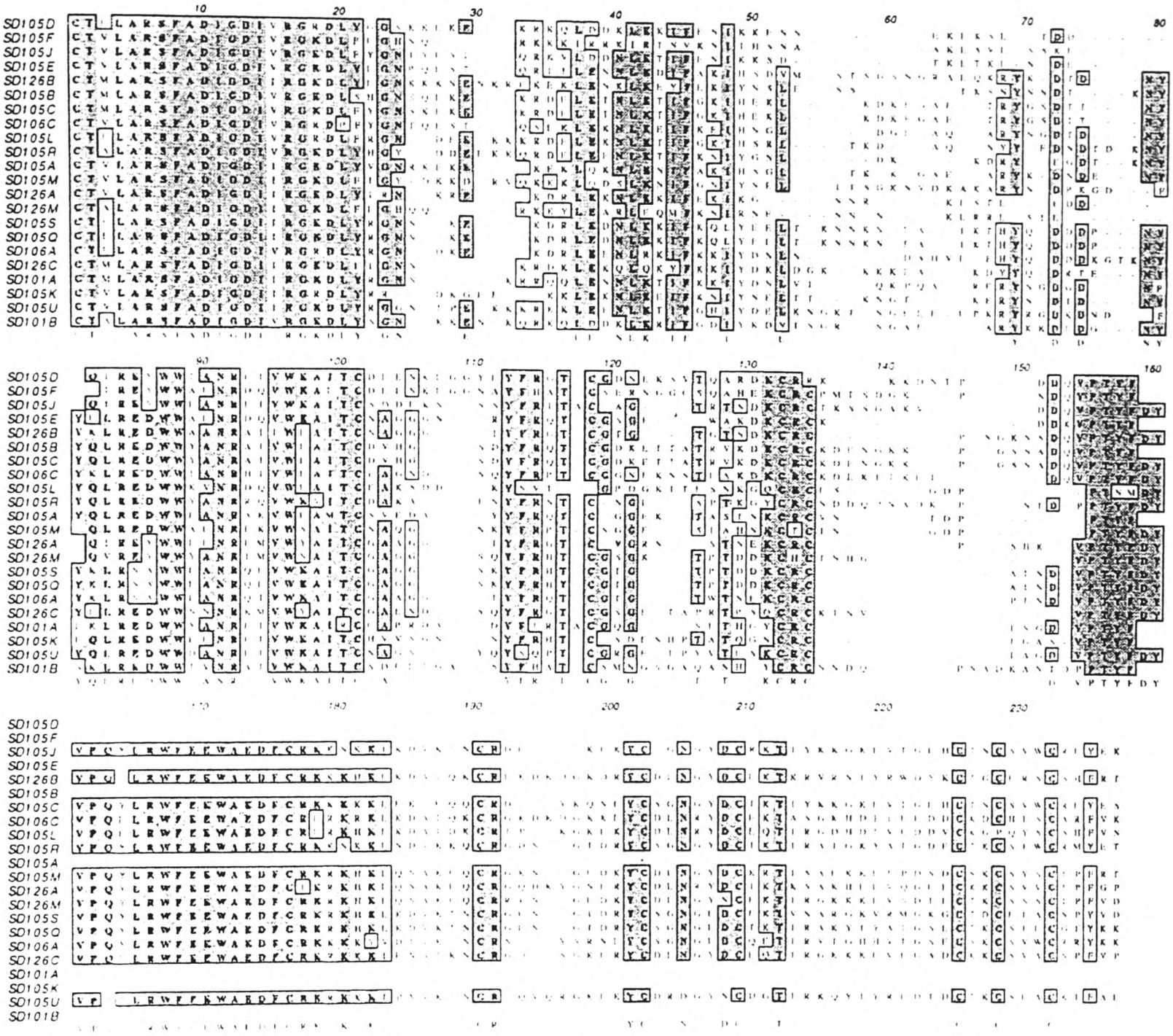

Fig. 3. Alignment of 22 Sudanese DBL-1 sequences from four parasite clones over the region between R1 and R2 (see Fig. 1). The first two residues of this alignment overlap with Fig. 2. Conserved residues are boxed and shaded dark grey; conservatively substituted residues are boxed and shaded light grey. Consensus residues are shown below the alignment.. The conserved cysteines at positions 1, 101, 118, 132, 134, 176, 190, 202, 209, 225, 228 and 232 correspond to cysteines 7–18, respectively. The aromatic residues phenylalanine, tyrosine and tryptophan are highly conserved at positions 8, 21, 45, 69, 80, 81, 87, 88, 96, 112, 113, 157, 158, 160, 164, 167, 168, 171, 176, 201, 207 and 235. The sequences SD105Q and SD105S are identical until position 209 after which they diverge.

3.2. DBL-1 domain variation between different var gene loci in the 3D7A clone

To analyse PfEMP-1 variation in a single clone, reverse transcription of clone 3D7A mRNA followed by PCR amplification and subcloning was used to obtain expressed PfEMP-1 sequences from a laboratory isolate, 3D7A, currently being sequenced by the *P. falciparum* Genome Project [21]. The sequence of 3D7A Chromosome 2 has recently been reported [22] and it contains two sub-telomerically located full-length *var* genes in addition to five small ORFs with sequences resembling the *var* exon II-related Pf60.1 genes which lack DBL-1 domains [23]. Asynchronous in vitro cultures of this clone express numerous different *var* genes. To date 25 different expressed PfEMP-1 cDNA sequences have been identified. Alignments have been made of these 25 sequences with 20 DBL-1 sequences from Genebank and the 22 Sudanese sequences over the region corresponding to positions 14–102 in Fig. 3 (data not shown). The patterns of variation in these sequences are essentially identical to that found in the Sudanese sequences analysed above and in an earlier comparative analysis of a smaller number of sequences [18]. Several large sequence blocks are shared with other sequences in the global data bank. The sequence 3D7-93 is identical to the Sudanese sequence SD105J. 3D7-16 and SD105M have a single synonymous substitution. Other 3D7A sequences are very similar to some Sudanese sequences. For example, 3D7-111 and SD105L (73/74 identical amino acids); 3D7-11 and SD105R (69/71); 3D7-3 and SD105F (54/63, with the 9 differences occurring in consecutive residues); and 3D7-44 with SD105E (66/78, all differences within a 32 residue block).

3.3. Phylogenetic analysis

Phylogenetic analysis was performed to investigate the relationship between the 56 Sudanese sequences and their relationship to 47 'global' DBL-1 sequences. Trees were generated using the same region of the molecule as was used to align the Sudanese sequences presented in Fig. 2. Neither the Sudanese nor any other set of sequences form an isolated sub-group of distinct DBL-1 domains. A total of 34 of the Sudanese sequences and 17 'global' sequences formed clusters statistically supported by the bootstrap re-sampling method (100 re-samples). A cladogram illustrating the relationships between these sequences is presented in Fig. 4.

Sudanese sequences cluster with sequences from Vietnam (bootstrap values of 70–96%); Brazil (bootstrap values of 96–98%); Gambia (bootstrap values of 74–89%); Kenya (bootstrap value of 70%) and 3D7A (bootstrap values of 83–100%). Three Sudanese sequences are identical to 3D7A sequences in this region (SD105D and 3D7A-4; SD105Q/SD105S and 3D7A-2; SD105R and 3D7A-1). SD106D is identical to the Brazil-1 sequence. SD106A shares 53/54 amino acid residues with the Kenya-1 sequence and SD106G shares 51/57 amino acid residues with the Vietnam-4 sequence.

Certain sequences from different Sudanese parasite clones appear closely related and cluster together. Cluster I (bootstrap support of 96%) contains five different sequences from 5/6 of the Sudanese clones analysed (SD101A/SD102C, SD105N, SD106G, SD126G and SD126J) and the Vietnam-4 sequence. These sequences share around 80% of residues over this ~ 60 amino acid sub-region. Cluster II (bootstrap support of 86%) contains the closely related sequences SD105D, SD106B, Gambia-1 and 3D7A-4.

A further phylogenetic analysis was performed on the region corrsponding to positions 14–102 in Fig. 3 (data not shown). Included in this analysis were 25 expressed 3D7A sequences, 22 Sudanese sequences and 20 'global' database sequences. Sudanese and 3D7A sequences grouped together in six clusters in this analysis (bootstrap values of 83–100%). Two of the expressed 3D7A sequences grouped with a Brazilian sequence (66% bootstrap support). Around half of all the sequences used in these comparisons (22 Sudanese and 29 'global' sequences) did not resolve into any statistically supported cluster because they are no more closely related to any one sequence than they are to any other in the data set.

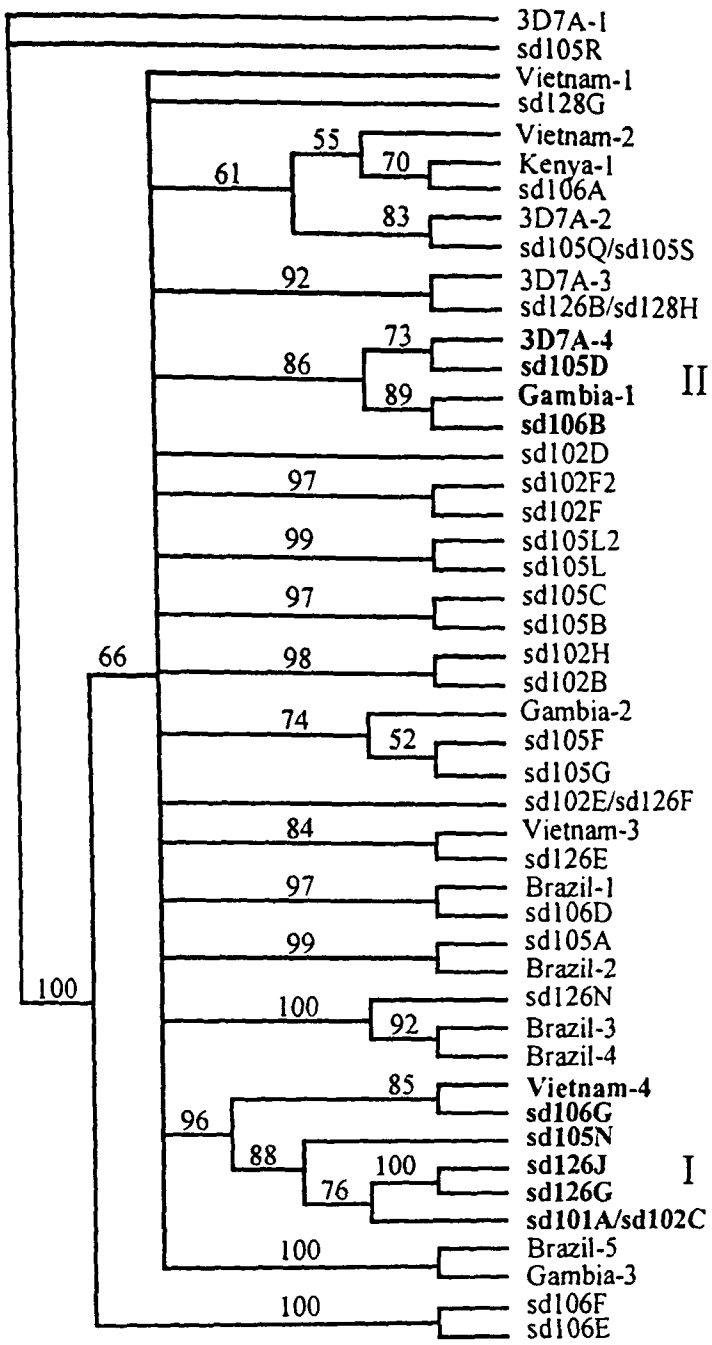

Fig. 4. A phylogenetic model of the relationship between 34 Sudanese and 17 Genebank sequences from diverse geographical locations. The model was generated by performing a Maximum Parsimony analysis using the PAUP program on a sequence alignment of the same region of DBL-1 as presented in Fig. 2. The alignment was made using the GCG and MacVector programmes and edited manually. Statistical support is provided by the booststrap resampling method (100 resamplings). Bootstrap values are shown above branch lines. Branches with a bootstrap value of less than 50% are collapsed. The non-Sudanese sequences have the following accession numbers 3D7A-1 (z94746); 3D7A-2 (z94751); 3D7A-3 (z94750); 3D7A-4 (z94749); Vietnam-1 (z94744); Vietnam-2 (z94737); Vietnam-3 (z94745); Vietnam-4 (z94741); Kenya-1 (z94730); Gambia-1 (u67960); Gambia-2 (af003473); Gambia-3 (u67959); Brazil-1 (l42245); Brazil-2 (y13408); Brazil-3 (y13402); Brazil-4 (l42247); and Brazil-5 (u31083). 22 Sudanese sequences and 31 sequences from the 'global' databases did not form statistically significant clusters with any of the other sequences analysed and are not included in this cladogram.

4. Discussion

The original sequence comparisons of PfEMP-1 variants showed that each protein had one to four domains with a sequence motif which had been noted in the Duffy positive erythrocyte binding domains of a merozoite surface protein of *P. vivax* and *P. knowlesi* [20]. This Duffy binding-like (DBL) motif has also been found in a *P. falciparum* erythrocyte-binding antigen (EBA 175) that binds erythrocyte surface sialic acid [24]. Even in the most conserved region of PfEMP-1, the DBL-1 domain, no more than 15–20% of amino acids are conserved in all variants and many thousands of different DBL-1 sequences must circulate in the parasite population's *var* genes. This study has analysed variation in this domain in sequences expressed by a single laboratory clone, in a set of clones derived from patients during the autumn 1989 malaria season in a Sudanese village and in the database of *P. falciparum* sequences.

Comparisons between these sequences show that there is marked conservation of the positions of certain residues in the DBL-1 domain. In particular, the positions of eight to nine pairs of cysteine residues are essentially invariant. The majority of DBL-1 sequences contain nine cysteine pairs although some variants lack one or two of these residues, or contain an additional cysteine. Positions of bulky aromatic residues such as tryptophan, tyrosine and phenylalanine are also conserved (as previously noted in related *Plasmodium* adhesion receptors [20,25]). The length of the hyper-variable regions between conserved motifs shows very little variation between different sequences. Large numbers of DBL-1 sequences share short (10–20 amino acid) regions of sequence including the one universally conserved sequence, the 11 amino acid LARSFADIGDI motif. Longer (20–80 amino acid) regions of homology can be identified in parasite clones isolated from patients in a Sudanese village, in isolates taken from infections in other areas of the globe and in the laboratory clone 3D7A.

These observations support a hypothesis that the structural framework of the DBL-1 domain in most, if not all, of these molecules is highly con-

served. Variants with unusual numbers of cysteines however, may form a subset with altered antigenic and adhesive properties. Possible selective constraints may be the requirement for binding to relatively invariant host receptors during erythrocyte invasion [20,24], or adhesion to uninfected red blood cells in the rosetting reaction [26,27]. Some mutations may be selected because they give rise to antigenic differences between functionally similar molecules. Structural diversity in PfEMP-1 DBL-1 domains seems less like that observed in the non-adherent variable trypanosome surface antigens and more analogous to the situation found in adhesion receptor-binding viruses such as Foot and Mouth Disease Virus (FMDV). In FMDV virions three diverse capsid proteins mask an essential RGD adhesin motif in the first virion protein which is essential for receptor binding and host cell invasion [28]. The LARSFADIGDI motif could have an analogous role in PfEMP-1 function.

Long stretches of amino acid identity occur between DBL-1 domains in the PfEMP-1 molecules of parasites from different locations and universally conserved motifs exist. Thus although the *var* genes potentially encode a vast amount of antigenic diversity, PfEMP-1 proteins appear to be composites of a set of more constrained sequences, presumably evolved from a common ancestral protein. Previous reports have differed on the extent to which the human agglutinating antibody responses to infected erythrocytes (considered to be mainly directed against PfEMP-1) are isolate specific [29,30]. In a recent study of agglutinating antibody specificity, isolates from infections in a Sudanese village were genetically characterised and their capacity to be recognised by sera samples from individuals from the same village was analysed [31]. Each of the isolates induced agglutinating antibodies capable of recognising at least some of the serotypes expressed by the other parasite isolates. Our data on PfEMP-1 diversity demonstrates that different parasite isolates can encode stretches of similar or identical PfEMP-1 sequence. It can therefore explain observations that agglutinating antibodies sometimes cross-react with geographically diverse isolates.

Generation of diversity by recombination has been suggested as the reason for the sub-telomeric chromosomal location of many *var* genes and their association with repetitive sequences [32]. Frequent recombination has been demonstrated in laboratory crosses of the parasite [16,33], and high rates of recombination have been observed in natural populations of *P. falciparum* [34]. A *P. falciparum* population model has proposed that individual PfEMP-1 variants can confer enhanced and long lasting immunity relative to sporozoite and merozoite antigens and will thus structure the parasite population into distinct and rarely recombining strains that have little overlap between their PfEMP-1 repertoires [13]. The phylogenetic analysis presented here, places *var* sequences from genetically different co-circulating parasite clones in clusters with a high bootstrap support. It also places some *var* genes from parasite isolates of diverse origin in well-supported clusters. Finally, it illustrates that many DBL-1 sequences are not more closely related to any particular sequence than they are to any other. Recombination between rapidly evolving sequences is the most plausible mechanism to explain these patterns of DBL-1 diversity. The phylogenetic analysis supports neither a 'distinct strains' model for *P. falciparum*, nor distinctly segregating repertoires of *var* genes. Rather, it adds to the accumulated evidence for frequent recombination in randomly mating populations of *P. falciparum*. [35,36]. Extra-cellular portions of PfEMP-1 other than DBL-1 are known to be immunogenic [37] and it remains possible to hypothesise that such epitopes could be sufficiently immuno-dominant to 'strain-structure' the parasite population. However these regions of the protein are even more polymorphic than the DBL-1 domain, and it is unlikely that they are exempt from the exchanges that appear to be shuffling and reshuffling DBL-1.

DBL-1 is only one of several distinct domains of the complex PfEMP-1 molecule and the other part of the conserved 'head structure', the CIDR domain, can bind CD36 without help from any other PfEMP-1 sequences [37,38]. Binding to thrombospondin, to CD36 and the rosetting of uninfected erythrocytes are the most common adhesive phenotypes of parasitised erythrocytes [39–

41]. Parasitised cells are known to express the cytoadherance phenotype prior to rosetting and immunoglobulin binding [42]. Whether CIDR-mediated endothelial adhesion precedes DBL-1-mediated red blood cell capture within capillary beds or whether sequestrin [39], other regions of PfEMP-1, or other variable parasite encoded erythrocyte membrane proteins [43,44] are involved in these processes is not known.

Acknowledgements

We thank Thor Theander, Lars Hviid, Trine Stalsoe, Haider Giha, Mercy Sowa, Alison Creasey, David Cavanagh, Chris Newbold and Alex Rowe for discussions. The work was supported by a Wellcome Prize Studentship to C.W., an MRC Training award to G.C., a Wellcome Senior Fellowship programme award to D.E.A. and an INCO-DC Project Grant from the European Union (IC18CT97-0238).

References

[1] Thaithong S, Beale GH. Fenton B, et al. Clonal diversity in a single isolate of the malaria parasite Plasmodium falciparum. Trans Roy Soc Trop Med Hyg 1984;78:242–5.

[2] Babiker HA, Creasey AM, Fenton B, Bayoumi RA. Arnot DE, Walliker D. Genetic diversity of Plasmodium falciparum in a village in eastern Sudan. I. Diversity of enzymes, 2D-PAGE proteins and antigens. Trans Roy Soc Trop Med Hyg 1991;85:572–7.

[3] Druihle P, Daubersies P, Patarapotikul J, et al. A primary malarial infection is composed of a very wide range of genetically diverse but related parasites. J Clin Invest 1998;101:2008–16.

[4] Felger I, Tavul L, Kabatnik S, et al. *Plasmodium falciparum*: Extensive polymorphism in an area with endemic malaria in Papua New Guinea. Exp Parasitol 1994;79:106–16.

[5] Beck H-P, Felger I, Huber W, et al. Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the Phase III trial of malaria vaccine Spf66. J Infect Dis 1997;175:921–6.

[6] Arnot DE. Clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission. Trans Roy Soc Trop Med Hyg 1998;92:580–5.

[7] Baruch DI, Pasloscke BL, Singh HB, et al. Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell 1995;82:77–87.

[8] Su XZ, Heatwole VM, Wertheimer SP, et al. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythroctes. Cell 1995;82:89–100.

[9] Roberts DJ, Craig AG, Berendt AR, et al. Rapid switching to multiple antigenic and adhesive phenotypes in malaria. Nature 1992;357:689–92.

[10] Smith JD, Chitnis CE, Craig AG, et al. Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell 1995;82:101–10.

[11] Marsh K, Otoo L, Hayes RJ, Carson DC, Greenwood BM. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans Roy Soc Trop Med Hyg 1989;83:293–303.

[12] Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat Med 1998;4:358–60.

[13] Gupta S, Day KP. A strain theory of malaria transmission. Parasitol Today 1994;10:476–81.

[14] Bayoumi RA, Creasey AM, Babiker HA, et al. Drug response and genetic characterisation of *Plasmodium falciparum* clones recently isolated from a Sudanese village. Trans Roy Soc Trop Med Hyg 1993;87:454–9.

[15] Bayoumi RA, Babiker HA, Arnot DE. Uptake and efflux of chloroquine by chloroquine–resistant *Plasmodium falciparum* clones recently isolated in Africa. Acta Tropica 1994;58:141–9.

[16] Walliker D, Quakyi IA, Wellems TE, et al. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science 1987;236:1661–6.

[17] Arnot DE, Roper C, Bayoumi RA. Digital codes from hypervariable tandemly repeated DNA sequences in the *Plasmodium falciparum* circumsporozoite gene can genetically barcode isolates. Mol Biochem Parasitol 1993;61:15–24.

[18] Kyes S, Taylor H, Craig A, Marsh K, Newbold C. Genomic representation of var gene sequences in *Plasmodium falciparum* field isolates from different geographical regions. Mol Biochem Parasitol 1997;87:235–8.

[19] Swofford DL. PAUP: phlyogenetic analysis using parsimony, version 3.1. Champaign, IL: Illinois Natural History Society, 1993.

[20] Chitnis CE, Miller LH. Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in eythrocyte invasion. J Exp Med 1994;180:497–506.

[21] Dame JD, Arnot DE, Bourke PF, et al. Current status of the *Plasmodium falciparum* Genome Project. Mol Biochem Parasitol 1996;79:1–12.

[22] Gardner MJ, Tettelin H, Carucci DJ, et al. Chromosome 2 sequence of the human malaria parasite *Plasmodium falciparum*. Science 1998;282:1126–33.
[23] Bonnefoy S, Bischoff E, Guillotte M, Mercereau-Puijalon O. Evidence for distinct prototype sequences within the *Plasmodium falciparum* Pf60 multigene family. Mol Biochem Parasitol 1997;87:1–11.
[24] Sim BKL, Chitnis CE, Wasniowska K, Hadley TJ, Miller LH. Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. Science 1994;264:1941–4.
[25] Adams JH, Sim BKL, Dolan SA, Fang X, Kaslow DC, Miller LH. A family of erythrocyte binding proteins of malaria parasites. Proc Natl Acad Sci USA 1992;89:7085–9.
[26] Chen BQ, Barragan A, Fernandez V, et al. Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) as the rosseting ligand of the malaria parasite *P. falciparum*. J Exp Med 1998;187:15–23.
[27] Rowe JA, Moulds JM, Newbold CI, Miller LH. *P. falciparum* rossetting mediated by a parasite variant erythrocyte membrane protein and complement-receptor 1. Nature 1997;388:292–5.
[28] McKenna TS, Rieder E, Lubroth J, Burrage T, Baxt B, Mason PW. Strategy for producing new foot and mouth disease vaccines that display complex epitopes. J Biotech 1996;44:83–9.
[29] Marsh K, Howard RJ. Antigens induced on erythrocytes by *P. falciparum*: Expression of diverse and conserved determinants. Science 1986;231:150–3.
[30] Newbold CI, Pinches R, Roberts DJ, Marsh K. *Plasmodium falciparum*: the human agglutinating antibody response to the infected red cell surface is predominantly variant specific. Exp Parasitol 1992;75:281–92.
[31] Giha HA, Staalsoe T, Dodoo D, et al. Overlapping antigenic repertoires of variant antigens expressed on the surface of erythrocytes infected by *Plasmodium falciparum*. Parasitology (in press).
[32] Thompson JK, Rubio JP, Caruana S, Brockman A, Whickham ME, Cowman AF. The chromosomal organisation of the *Plasmodium falciparum var* gene family is conserved. Mol Biochem Parasitol 1997;87:49–60.
[33] Kerr PJ, Ranford-Cartwright LC, Walliker D. Proof of intragenic recombination in *Plasmodium falciparum*. Mol Biochem Parasitol 1994;66:241–8.
[34] Conway DJ, Roper C, Oduola AMJ, et al. High recombination rate in natural populations of *Plasmodium falciparum*. Proc Natl Acad Sci USA 1999;96:4506–11.
[35] Babiker HA, Ranford-Cartwright LC, Currie D, et al. Random mating in a natural population of the malaria parasite *Plasmodium falciparum*. Parasitology 1994;109:413–21.
[36] Paul REL, Packer MJ, Walmsley M, et al. Mating patterns in malaria parasite populations of Papua New Guinea. Science 1995;269:1709–11.
[37] Baruch DI, Ma XC, Singh HB, Bi X, Pasloske BL, Howard RJ. Identification of a region of PfEMP-1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36:conserved function with variant sequence. Blood 1997;90:3766–75.
[38] Smith JD, Keyes S, Craig, et al. Analysis of adhesive domains from the A4VAR *Plasmodium falciparum* erythrocyte membrane protein-1 identifies a CD36 binding domain. Mol Biochem Parasitol 1998;97:133–48.
[39] Ockenhouse CF, Ho M, Tandon NN, et al. Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria; differential adhesion of infected erythrocytes to CD36 and ICAM-1. J Infect Dis 1991;164:163–9.
[40] Newbold CI, Craig AG, Kyes S et al. PfEMP-1, polymorphism and pathogenisis. Ann Trop Med Parasitol 1997;551–7.
[41] Rowe A, Obeiro J, Newbold CI, Marsh K. Plasmodium falciparum rosetting is associated with malaria severity in Kenya. Infect Immun 1995;61:2323–6.
[42] Treutiger CJ, Carlson J, Scholander C, Wahlgren M. The time course of cytoadhesion, immunoglobulin binding, rosette formation, and serum induced agglutination of *Plasmodium falciparum*–infected erythrocytes. Am J Trop Med Hyg 1998;59:202–7.
[43] Cheng Q, Cloonan N, Fischer K, et al. stevor and rif are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. Mol Biochem Parasitol 1998;97:161–76.
[44] Helmby H, Cavelier L, Petterson U, Wahlgren M. Rosetting *Plasmodium falciparum*-infected erythrocytes express unique strain-specific antigens on their surface. Infect Immun 1993;61:284–8.